METHYLMERCURY IN CAPTIVE DOUBLE-CRESTED CORMORANTS (*PHALACROCORAX AURITUS*): OCULAR ELECTROPHYSIOLOGY, TISSUE MERCURY DISTRIBUTION, PATHOLOGY, HEMATOLOGY, AND NEUROBEHAVIORAL ASPECTS

By

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For Gary

husband, partner, and best friend

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Methylmercury (MeHg), a persistent environmental neurotoxicant which can bioaccumulate up the food chain, poses a risk to piscivorous species such as cormorants. This study evaluated multiple parameters for use in detection of early indications of MeHg exposure including ocular electrophysiology, histopathology, hematology, tissue mercury (Hg) distribution, and behavioral and neurological testing.

Twenty-two ~8-week-old double-crested cormorants (*Phalacrocorax auritus*) were dosed for 8 weeks with one of three treatments: (A) control, (B) 3.5 mg, or (C) 0.5 mg MeHgCl/kg diet/day. Electroretinograms (ERGs) and visual evoked potentials (VEPs) were performed at 0, 4, and 8 weeks. Photopic and scotopic ERG profiles were recorded, a- and b-wave parameters were evaluated, and the Naka-Rushton model was applied to the profiles. Light-adapted VEP responses were recorded as signal-averaged waveforms.

Tissues were evaluated histologically and Hg was localized by autometallography. Retinal sections were further evaluated by electron microscopy. Mercury levels were determined by cold vapor atomic absorption spectrophotometry. Serum biochemistries, CBCs, weekly neurological examinations, and behavioral observations were evaluated.

In the high-dose group proprioceptive deficits were seen starting at week 5 and the VEP P1-wave amplitudes were decreased compared to controls on weeks 4 and 8. Other VEP and neurological parameters, ERGs, and most other clinical tests were unaffected. Neuropathology included swollen myelin sheaths and digestion chambers with debris in sciatic nerves, axonal degeneration in the spinal cord, diffuse gliosis, and swollen ependymal cells in the brain. The high-dose birds averaged 11.1 μg/g and 58.5 μg/g of Hg in brain and liver, respectively. Ocular histological changes were found in the nerve fiber layer, outer plexiform layer, inner plexiform layer, and photoreceptor outer segments. Mercury was localized in these same retinal layers and in nonpigmented ciliary epithelium. Pronounced Hg staining in the brain was seen in the nucleus isthmi complex, optic tectum, other nuclei of the midbrain and medulla, and Purkinje cells.

VEPs and proprioception were useful in detecting early preclinical effects of MeHg in cormorants at 4-5 weeks of dosing at the 3.5 ppm dosing level, while ERGs and Naka-Rushton parameters were not. A level of 3.5 mg MeHg/kg diet/day may pose a risk of neurotoxicity to cormorants, especially for a duration of greater than 4-5 weeks. There was no evidence of toxicity at the 0.5 mg/kg level for 8 weeks; however, the potential for subtle effects on reproduction or reproductive behavior, which have been reported at low exposure levels in other avian species, have yet to be evaluated in cormorants.

CHAPTER 1
INTRODUCTION

Methylmercury (MeHg) is a well known potent neurotoxicant which persists in the environment, accumulates in tissues, and is very slowly eliminated from the body. The toxic effects of MeHg are insidious, chronic, and usually permanent. Certain regions in the United States, such as the Great Lakes and Florida, are known to have high mercury (Hg) concentrations which may present a significant risk to human and wildlife populations, especially those that consume substantial quantities of seafood. Mercury also continues to be a worldwide problem, due largely to global atmospheric cycles.

In 1991 the Florida Department of Health and Rehabilitative Services (1993) published a health advisory recommending restricted consumption of certain fish in many parts of Florida, due to their relatively high mercury content of 0.5 to1.5 ppm, especially large areas of south Florida and the Everglades. The department also recommended no consumption of certain fish with mercury levels above 1.5 ppm. Recently, osprey (Wiemeyer et al., 1987) and a variety of wading birds (Sundlof et al., 1994) have been found to have elevated tissue mercury levels. South Florida and Everglades cormorants have also been found to have high liver and brain levels of Hg (Sepúlveda et al., 1998).

In humans (Sabelaish and Hilmi, 1976), non-human primates (Merigan et al., 1983), and several other species (Gitter et al., 1988; Mattsson et al., 1981; Gramoni, 1980) disturbance of the visual system is often an early symptom of MeHg intoxication.

Visual dysfunction can include blurred vision, constriction of the peripheral visual fields, and blindness in severe cases. Other neurological affects of MeHg can include paresthesia, tremors, ataxia, sensory loss, memory loss, and, in the worst cases, coma and death.

In avian species it has been shown that birds will also develop neurological symptoms when exposed to methylmercury, but the effects on the visual system have not been well characterized. Sight in avian species is of concern due to their high degree of reliance on vision to survive. Avian species have a highly developed visual system, exemplified by extremely large eyes in proportion to their skull size, specialized retinal structures, and large areas of the brain dedicated to visual processing. Predatory birds in particular are critically reliant upon their visual perception due to the requirement of precise coordination in locating, tracking, and capturing rapidly moving targets. Among the neurological effects of MeHg, the effects on the visual system could potentially pose an incapacitating problem for species so highly dependent on sight. The use of the electroretinogram (ERG) and visual evoked potential (VEP) for detection of subclinical toxicities has been applied in humans and in other species, but has not been investigated in wild aquatic avian species in a systematic manner.

Avian species do not readily show signs of illness until they become critically decompensated; it is essential to devise a means by which to detect the subtle effects of toxicants such as mercury before a critical threshold is reached. The tests proposed here have the potential of enhancing our understanding of the effects of MeHg on free-ranging wildlife, benefitting endangered and protected species, and providing tools with which to monitor animals in a noninvasive manner.

The purpose of this project was to study the neuro-ophthalmological effects of methylmercury exposure in a wild avian species under controlled conditions and to evaluate whether sensitive electrophysiological tests, such as ERGs and VEPs, could be used to detect early changes related to methylmercury exposure. In conjunction with the ophthalmologic and electrophysiologic examinations, neurological and behavioral observations, hematologic and histopathologic measurements, and tissue distribution of mercury were evaluated to provide a thorough characterization of possible exposure responses.

The hypothesis of this project was that subclinical neuro-ophthalmological effects of MeHg exposure in cormorants are detectable by sensitive electrophysiological testing, including electroretinograms and visual evoked potentials, and that these changes will correlate to total Hg concentrations in specific tissues. The goal was to develop a sensitive technique to detect subclinical visual and/or neurologic effects of MeHg exposure in an avian species which is potentially at risk in the wild. Double-crested cormorants provided a useful model, representing an abundant and accessible fish-eating avian species which can acclimate reasonably well to handling and captivity.

Objectives

1. To establish baseline parameters for ERGs and VEPs and characterize the ERG b-wave amplitude response profile using the Naka-Rushton equation.
2. To analyze the effects of three levels (control, 0.5 ppm, and 3.5 ppm) of daily dietary methylmercury on ERG and VEP parameters in double-crested cormorants.

3. To characterize hematologic, neurologic, ophthalmologic, and behavioral changes resulting from MeHg exposure and to relate these to mercury levels in various tissues.

4. To characterize selected histological, histochemical, and ocular ultrastructural changes in MeHg exposed birds.

A variety of other electrophysiological tests – such as electromyograms, auditory and somatic evoked potentials, and nerve conduction velocities – could have been selected as test endpoints. However, it was my desire to study the ophthalmologic aspects of MeHg toxicity; hence the focus on ERGs and VEPs. Furthermore, as with many research projects, additional questions presented themselves as the investigation progressed. The logistical effort, time, and expense of capturing and caring for wild birds in captivity are considerable. In an effort to utilize the situation in the best way, and give as much meaning to the sacrifice of the birds as possible, many extra tissue samples and other data were collected. The main components of the project, as defined in the objectives, are presented in the chapters that follow. Data, samples, and satellite projects that evolved as the study proceeded, but which were not within the scope of this dissertation, will be used for future research.

CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

Historical Perspective

The unique properties of mercury – such as its fluidity, high surface tension, poor conductivity, and ability to alloy with other metals – have provided many varied applications of this metal in industry and medicine. Knowledge of mercury dates back to antiquity and was mentioned by early scholars such as Theophrastus of Erebus (370- 287 B.C.) and Pliny the Elder (A.D. 23-79) (Goyer, 1996). Paracelsus (1493-1541) introduced mercury as a treatment for syphilis, and contributed, after Ellenberg (circa 1480), to publications on the hazards of mercury and lead in goldsmithing (Gallo, 1996).

In the late 19th century use of mercury nitrate in the fur-felt hat industry caused occupational poisonings, and signs of this condition may have been the origin of the phrase "mad as a hatter" (Chang, 1980). Prior to the 20th century most incidents of mercury poisoning were due to inorganic salts. Introduction of organic mercury salts for the control of fungal infections, as well as increasing industrial uses, led to more cases of exposure and toxicity. In 1940 four cases of methylmercury toxicity in humans were reported as well as experimental toxicity in early studies of rats and a monkey (Hunter et al., 1940). In 1954 Hunter and Russell described detailed histopathology of the previous cases, and as a result the condition was referred to as Hunter-Russell syndrome.

The first large-scale "epidemic" of organomercurial poisoning occurred in Japan, in the 1950s, due to the discharge of metallic mercury-contaminated industrial waste into Minamata Bay. The aquatic microorganisms in the bay converted the metallic mercury into methylmercury which then accumulated in fish and shellfish. Hundreds of local residents suffered severe and often fatal neurological, reproductive, and developmental toxicity due to consumption of the contaminated fish. The source of mercury was traced to untreated discharge from a chloralkali plant. Similar events occurred in Iraq, which had several severe epidemics from the 1950s to the 1970s, where thousands were affected due to the ingestion of grains treated with organomercurial antifungal agents.

Through the bans on some uses of mercury, such as in seed dressings, wood preservatives and paints, the reduction of Hg use in pharmaceuticals, and the implementation of improved recycling programs, mercury pollution and exposure risks have been reduced. At present, the most common route of human exposure to organic mercury is ingestion of contaminated food sources, especially fish. By comparison, inorganic mercury remains primarily an occupational risk. In the past, many species of birds were at risk of exposure to treated grains, but, as with people, avian exposure risk is now mainly from the aquatic food chain.

Sources, Forms, and Cycles of Mercury

Mercury is a ubiquitous and naturally occurring element in the earth's crust. It is released into the environment by natural degassing and erosion as well as by anthropogenic processes. Human activities and uses which contribute to the circulating

mercury include combustion of fossil fuels, smelting, mining, waste incineration, medical and pharmaceutical laboratories, the electrical industry, pulp and paper production, and agriculture (ATSDR, 1994; Goyer, 1996).

Mercury exists in three valence states – Hg^0, Hg^+, and Hg^{2+} – and may be found as metallic mercury or a variety of organic or inorganic complexes. The global mercury cycle involves the release of mercury into the atmosphere, through degassing or other processes, atmospheric transport, mainly as elemental mercury, and then deposition back to ground and water (Goyer, 1996). More localized distribution can be caused by point sources such as release into bodies of water. Environmental transformation can occur among the various forms of mercury by way of oxidation and reduction reactions, methylation and demethylation reactions, or photolysis of organic mercurials (ATSDR, 1994).

Methylmercury, which is the most toxic form of mercury, has the greatest bioavailability among the different forms and can bioaccumulate up the food chain. The methylation transformation is, therefore, of primary importance when considering mercury toxicity. Under appropriate conditions any form of Hg entering an aquatic system can potentially be converted to MeHg. Various soil and aquatic microorganisms, especially sulfur-reducing bacteria, are capable of methylating inorganic Hg, under both aerobic and anaerobic conditions, in a process which is likely mediated non-enzymatically by a bacterial methyl cobalamine compound (Regnell and Tunlid, 1991; Gilmour and Henry, 1991). Various factors may affect the transformation of mercury including low pH and anaerobic conditions which both favor methylation, and high levels of dissolved organic carbon which reduces methylation (Gilmour and Henry, 1991; Regnell and Tunlid, 1991).

Absorption, Distribution, and Excretion

The predominant route of exposure of animals or humans to MeHg is by ingestion. In humans gastrointestinal absorption of organic mercury is 90-95%, compared to only about 7% for inorganic salts (Goyer, 1996). Little data is available on specific gastrointestinal absorption rates of MeHg in birds. Data on retention of ingested MeHg over various time frames indicate that retention is generally very high, although dose, tissue type, and age-related variables exist (March et al., 1983; Lewis and Furness, 1991). Charbonneau et al. (1976) showed that there was no difference in the bioavailability of MeHg to adults cats fed either a diet of uncontaminated fish with pure MeHgCl in corn oil added or a diet of methylmercury-contaminated fish.

Methylmercury, due to its lipophilic nature, crosses membranes easily and is distributed readily to most tissues including brain, fetus, and eggs in birds. In birds the liver often accumulates the highest concentration of methylmercury, but this can be species dependent, as in some birds the kidney accumulates more than the liver (Scheuhammer, 1988; Miller et al., 1970; Lewis and Furness, 1991). In humans and mice, kidneys contain the greatest concentrations of mercury after exposure to organic mercury compounds (Ryan et al., 1991; Rice, 1989). The relative affinity of mercury compounds for the CNS is the greatest for organic mercury, followed by mercury vapor and inorganic mercury. Because of the easy passage of methylmercury into other organs and its even distribution in tissues, blood concentrations are considered a good indicator of tissue concentration in a dose-independent fashion (Norberg, 1976). In mammals all forms of mercury can cross the placenta, and there is higher toxicity to the fetus due to an enhanced fetal uptake.

Excretion of MeHg in humans, and in most animals, is mainly by way of the feces (biliary route) and to a lesser extent in urine (Norseth and Clarkson, 1970). Excretion varies depending on the form, dose, and time after exposure. Half-lives vary for different compounds and for different species. In humans the half-life of methylmercury is approximately 70 days, while for inorganic salts and elemental Hg the half-life is about 40 days and 35-90 days, respectively (ATSDR, 1994).

In birds excretion is also through fecal-urate material, although feathers have also been shown to be an important route of elimination of mercury (Honda et al., 1986; Braune, 1987; Furness et al., 1986). Eggs from exposed females can also contain significant amounts of mercury (Burger, 1994; Fimreite, 1971), which can potentially pose an important hazard to reproductive success. The MeHg half-life in birds is also variable and ranges from about 35 to 90 days (Soares et al., 1973). Soares et al. (1973) also reported that in chicks the biological half-life increased with increasing dietary MeHg levels. Fecal excretion in that study was about 0.66 - 1.0 % per day during a depletion period, and slightly higher excretion rates were measured with longer dosing.

Biotransformation, Metabolism, and Mechanisms

Once absorbed and in the tissues, methylmercury can be converted into divalent mercury (Hg^{2+}) (Dunn and Clarkson, 1980), most likely in liver and kidney (Lind et al., 1988; Magos et al., 1976; Norseth and Clarkson, 1970). Studies have shown that rat liver microsomes can convert MeHg to inorganic mercury and that this process may be related to hydroxyl radical production which is formed by microsomal cytochrome p-450

reductase (Suda and Hirayama, 1992). Suda and Takahashi (1992) also found that hypochlorous acid scavengers can also convert MeHg to inorganic mercury.

The brain, in contrast to liver and kidney, will contain mainly methylmercury for long periods. Conversion from MeHg to Hg^{2+} states in the brain can occur as shown in chronic Minamata cases where, after 20 years or more of methylmercury exposure, the predominant form of mercury in the brain was divalent mercury (Takeuchi, 1989). There is no indication that any organic forms of mercury are formed within mammalian tissues. However, in some species methylation (Rudd et al., 1980) and to some extent demethylation (Rowland et al., 1980) have been shown to be performed by intestinal microbes.

Mercury is known to bind sulfhydryl and disulfide groups and thus has the ability to bind proteins and enzymes diffusely throughout a system. These types of reactions can lead to nonspecific cell injury and death and subsequently may affect a wide variety of tissues and systems. Once absorbed, mercury can form soluble complexes with the sulfhydryl-rich molecules cysteine and glutathione (Aschner and Aschner, 1990). These complexes are secreted in the bile and undergo enterohepatic recirculation (Ballatori and Clarkson, 1982; Norseth and Clarkson, 1971; Berlin et al., 1975). Organic mercurials may also bind specific receptor sites that inhibit sodium transport, as is thought to occur with certain mercury based diuretics.

It is still uncertain whether methylmercury or the mercuric ion is responsible for the neurotoxic effects, although it has been suggested that the mechanisms of toxicity of inorganic and organic mercurials are similar and that differences in toxicity are related to

differential accumulation in sensitive tissues (ATSDR, 1994). Differences do exist, however, in the nature of the interaction of inorganic and organic mercury on a molecular level, as is exemplified by the ability of mercuric mercury, but not methylmercury, to induce synthesis of metallothionein, a metal-binding protein, in kidney cells (Madsen and Christensen, 1978; Goyer, 1996).

A variety of mechanisms for mercury toxicity have been proposed including formation of reactive oxygen species (Yee and Choi, 1996; Ali et al., 1992), depletion of antioxidants (Yee and Choi, 1996), inhibition of protein synthesis (Yoshino et al., 1966a; Cheung and Verity, 1981; Omata et al., 1978), enzyme inhibition (Paterson and Usher, 1971; Salvaterra et al., 1973), inhibition of oxygen uptake, and interference with mitochondria and the electron transport system (Yee and Choi, 1996). Additional mechanisms may include inhibition of ion channels (Sirois and Atchison, 1996; Leonhardt et al., 1996), altered divalent cation homeostasis, especially calcium (Denny and Atchison, 1996), and interference with the microtubular structure and axonal transport mechanisms (Aschner et al., 1987; Aschner, 1987). Regarding the propensity of MeHg to enter the nervous system, it has generally been considered that methylmercury is more lipophilic than inorganic forms of Hg and that this accounts for the ability of MeHg to diffuse across membranes and to cross the blood-brain barrier (BBB). A potential carrier-mediated mechanism has been studied in which MeHg is thought to form a cysteine conjugate that can mimic methionine and cross the BBB by way of methionine transporter (Aschner and Aschner, 1990; Kerper et al., 1992).

Tissue Specificity of Mercury Toxicity

The reasons for the selective toxicity of mercury to CNS are not entirely clear. MeHg is more toxic to the CNS than inorganic mercury, and this is thought to be due primarily to the ability of MeHg to cross the blood-brain barrier. The metabolic requirements of different tissues may also play a role in selective toxicity. The CNS has high oxygen and energy requirements and is particularly sensitive to disruptions in their supply. Mercury has been reported to inhibit oxidative phosphorylation and possibly to reduce oxygen uptake by neurons (Yee and Choi, 1996). The sensitivity to energy requirements may predispose nervous tissue to damage. Furthermore, lower levels of glutathione and metallothionein in the brain may also contribute to the availability of unbound Hg which could bind to potentially critical sites. Once MeHg crosses the BBB, it is believed that demethylation may occur and that this may contribute to the retention of mercury in the CNS since it would no longer be able to cross the BBB easily. Thus, the CNS may be exposed to a low dose but be affected for a prolonged period of time.

The liver tends to accumulate high concentrations of mercury when methylmercury is ingested. This occurrence is probably the result of several factors including the first-pass effect in which the intestinal blood supply drains through the liver where toxicants can be removed. The liver contains high concentrations of proteins and enzymes, including glutathione and cysteine, with which mercury can complex. The liver also contains high levels of other sulfur-rich molecules such as metallothionein, a metal-binding protein, which may serve as a "sink" for the metal by providing a substance to which it can bind and preventing its binding to more sensitive cellular components.

A key element in the toxic process is the ability of tissues to repair and/or compensate for damage. The liver has the ability to replace damaged or dying cells, whereas the nervous system cannot. The cellular targets of mercury could also affect components in the neurons which critically affect their function, but the same target in a liver cell may not have the same impact on that cell as it does on a nerve cell.

Toxicity of Methylmercury

Of the various forms of mercury, MeHg is the most important in terms of health effects due to environmental exposure. Methylmercury is considered primarily a neurotoxicant, as well as a significant reproductive and developmental toxicant. The neurotoxicity of MeHg has been consistently observed in many species, although the details of the neurological effects may vary somewhat. The following sections summarize the present knowledge of MeHg toxicity in avian species and provide a comparison with other species.

Neurotoxicity

Avian species

Numerous studies have been conducted on the effects of various mercurial compounds in a wide variety of avian species. The majority of experimental data has been derived from domesticated species or wild species that are herbivorous. The highest risk groups of free-ranging birds are currently carnivorous species, especially fish-eating species. Much of the mercury data on this group of birds is limited to the collection of

field data on mercury levels. Due to species variability, numerous environmental factors that would affect the status of these birds, and scant experimental data in comparable species, conclusions about much of the field findings are difficult to make.

Birds affected by methylmercury exhibit signs of weakness, incoordination, and ataxia. Birds become lethargic, have fluffed feathers, difficulty balancing, flying and landing (Scheuhammer, 1988; Swensson and Ulfvarson, 1969; Borg et al., 1970; Fimreite and Karstad, 1971; Pass et al., 1975; Finley et al., 1979). High exposure levels may also result in head tremors, paralysis, and seizures progressing to coma and death. A variety of other signs have been reported in birds including decreased food intake (Borg et al., 1970; Bhatnagar et al., 1982; Hill and Soares, 1987), weight loss (Fimreite and Karstad, 1971; Swensson and Ulfvarson, 1969; Bhatnagar et al., 1982), and reproductive and behavioral effects (Fimreite, 1971; Heinz and Locke, 1976; Barr, 1986). Reports on the effects of MeHg on vision in birds are very limited. Vision in birds is difficult to evaluate, which has left this aspect of the toxicity relatively unexplored, especially in wild avian species.

Onset of signs is variable and depends on species, form of mercury, dose, duration, route of administration, and selenium levels. Selenium has been shown to have a protective effect against mercury toxicity (Chang, 1982; Skerfving, 1978; Ganther et al., 1972; Magos et al., 1987; Stoewsand et al., 1974). In general, it is considered that tissue concentrations of mercury are good indicators of the likelihood of a bird succumbing to MeHg toxicity, with brain levels of 10-15 ppm, and liver levels of greater than 30-40 ppm considered potentially lethal (Scheuhammer, 1988). There is general agreement in the literature that when birds begin to exhibit overt clinical signs of methylmercury

neurotoxicity they will eventually die, even if no more mercury is given (Hill and Soares, 1987; Scheuhammer, 1988). Examples of some of the experimental studies in which various avian species were fed methylmercury are summarized below.

Great egret nestlings dosed at 5.0 mg/kg dietary MeHg exhibited severe neurological signs after 8 weeks of dosing (Frederick et al., 1997). Decreases in food intake and body weights were observed in that study in the high (5.0 mg/kg) and the low (0.5 mg/kg) dose levels. Blood mercury in the great egret nestlings remained low for the first few weeks, and at about 5 weeks of dosing levels rapidly increased. This finding was proposed to be related to feather growth, as the increase in blood Hg corresponded to cessation of feather growth (Frederick et al., 1997).

Zebra finch fed 5.0 μg/g dietary methlymercury chloride developed typical neurotoxicity including lethargy, fluffed feathers, and difficulty flying, landing, and balancing on perches, as early as day 40 of dosing (Scheuhammer, 1988). In the same experiment, birds dosed with only 1.0 or 2.5 ug/g dietary MeHg showed no signs of intoxication throughout 76 days of dosing.

Borg et al. (1970) fed goshawks contaminated chickens which had been fed with MeHg-treated wheat. Three goshawks which were fed chicken with about 13 ppm, and one fed about 10ppm, died within 30-47 days. The birds exhibited a period of balance disturbances, weakness, staggering, and incoordination which progressed to ataxia and complete immobility. At day 10 of dosing food consumption decreased, and neurological signs developed between 11 and 17 days. A change in temperament (calming of normally furious temper) was noted as early as day 8.

Fimreite and Karstad (1971) fed red-tailed hawks a diet of chick livers from chicks that had been fed methylmercury dicyandiamide (MMD)-treated seed for 4 or 12 weeks. Some, but not all, of the hawks fed at a rate of 10 mg Hg/kg liver developed neurologic signs including weakness of the extremities, uncoordinated movements, and difficulty eating, and they eventually died. Signs first appeared at about 4 weeks. Another bird fed 7.2 mg Hg/kg liver also died, but signs developed more gradually. At the 3.9 mg/kg level of dosing, no clinical signs were detected. Liver Hg levels in birds that died were ≥20 ppm (Fimreite and Karstad, 1971).

A study of starlings, grackles, red-winged blackbirds, and cowbirds found that grackles were the most sensitive species of the group when fed 40 ppm MeHg (Finley et al., 1979). Sensitivity was gauged by the number of days of dosing required for 5 of 14 birds to die. This endpoint was reached in 6 days for grackles, 8 for starlings, 10 for cowbirds, and 11 days for redwings.

Bhatnagar et al. (1982) determined that Pekin ducks fed 15 ppm MeHg developed neurological signs starting at week 5 of dosing. Signs were similar to other avian studies and included decreased mobility, refusal of food, leg paralysis, and, in severe cases, convulsions. Birds dosed at 0.5 and 5.0 ppm MeHg showed no neurological signs.

Day-old White Rock chicks were fed methylmercuric chloride for 4 or 7 weeks, at 6 different doses (Soares et al., 1973). At 16.88 ppm all chicks died by day 17, and at 7.8 ppm all died by 21 days. Even at the 5.09 ppm level, 50 % died by day 33. Neurological signs were consistent with those described previously. The earliest onset of symptoms was at day 9 (16.88 ppm), and the progression to death often occurred in as little as 3 days. Chicks fed 0.50, 1.02, or 2.19 ppm were not affected.

In contrast to the acute or subchronic dosing studies above, Heinz (1979) studied reproductive and behavioral effects in three generations of mallard ducks fed 0.5 ppm for three years. There were no neurological signs of intoxication or deaths in any of the adults fed at this level. The levels of mercury in tissues of adult birds in this study remained relatively constant over the three generations, and body weight did not change. However, reproductive and behavioral effects occurred and will be discussed in a later section.

Humans

Reports of organic mercury poisoning in humans (Minamata Bay, Japan, and Iraqi epidemics) described a neurological syndrome with a multitude of symptoms including, paresthesia, impaired peripheral vision or blindness, deficits in hearing, taste and smell, slurred speech, unstable gait, ataxia, muscle weakness, irritability, memory loss, depression, and sleeping difficulties (Chang, 1982; Igata, 1993). In addition to the above list, Iraqi victims were observed to have cerebellar ataxia, spasticity, abnormal reflexes, confusion, hallucinations, excitement, and loss of consciousness (ATSDR, 1994). Severity of some signs were related to the dose and duration of MeHg intake. The presentation and subsequent pathological findings in the two epidemics varied slightly, presumably due to the different nature of exposure (relatively acute vs. chronic). In Japan, contaminated fish were consumed over a period of many years, while in Iraq the exposure was from consumption of contaminated grain for several months and no residual or chronic symptoms have been reported (Igata, 1993).

The primary neuropathologic changes associated with MeHg intoxication in humans and primates occur in the cerebral cortex and cerebellum, characterized by focal

necrosis of neurons, lysis and phagocytosis, followed by proliferation of glial cells (Evans et al., 1977; Shaw et al., 1975). The deeper sulci such as the visual cortex and insula are preferentially affected. Acutely, there may be cerebral edema, but chronically, degeneration of grey matter leads to cerebral atrophy (Chang, 1980; Takeuchi, 1985).

Children exposed *in utero* to organic mercury may also have a range of symptoms depending on the duration, dose, and time of gestation at which they were exposed. Severe MeHg intoxication of pregnant women may lead to fetal death. Lower levels of exposure may cause varying degrees of cerebral palsy and mental retardation. Although an infant exposed *in utero* may appear normal at birth, they can develop psychomotor deficits as they mature (Choi, 1989).

Experimental and domesticated species

Despite variations in species and compounds used in many experimental mercury intoxications, the primary neurotoxicity of methylmercury is consistent and resembles conditions documented in human cases (Chang, 1982). Acute MeHg toxicity in monkeys resulted in sudden onset anorexia, gross ataxia, abnormal posture, with preservation of fine movement (Shaw et al., 1975). Chronic exposure resulted in subtle behavioral changes, loss of fine digital control, ineffective chewing and anorexia (Shaw et al., 1975). Rats and mice presented typical neurotoxic symptoms including muscle spasms, gait disturbances, flailing, and hind limb crossing (Møller-Madsen, 1991; Somjen et al., 1973).

In cats dosed at 0.25 mg/kg/day, the first sign of toxicity was weakness of the hind legs at about 77 days (Charbonneau et al., 1974). Toxicity progressed to include ataxia, hind leg rigidity, wide stance, high stepping, and intention tremors. Impaired

righting reflex and convulsions also developed with continued exposure. In a chronic study in cats fed a tuna diet containing 0.5 ppm, similar neurologic signs appeared after 7-11 months (Chang et al., 1974). Charbonneau et al. (1974) also found that there was no difference in the time of onset of toxicity between those cats which were fed MeHg-contaminated fish and those which were dosed with MeHg added to uncontaminated fish.

In a subacute study of MeHg toxicity in swine, all animals developed neurotoxicity after ingesting 1.29, 0.86, 0.64, and 0.43 mg/kg of body weight daily (Davies et al.,1976). Ataxia, dysmetria, blindness, convulsions, paresis, and death were reported. Prior to the onset of signs, a reduction in feed consumption was noted, and the onset time was longer as the dose decreased.

Aquatic species and other wildlife

The nature of the environment in which fish live alters their risk to exposure. Both inorganic and organic mercury compounds are toxic to fish. Organic mercury is more toxic to fish than inorganic. Environmental factors such as temperature, salinity, oxygen levels, pH, and water hardness can affect the relative accumulation and toxicity of methylmercury to aquatic organisms (Spry and Wiener, 1991). Much of the literature on toxicity of mercury to fish has focused on lethality and reproductive effects as end points. Behavioral studies indicate that various mercury compounds in solution may impair behavioral-avoidance responses in fish (WHO, 1989).

In a study of Florida largemouth bass ($\geq$ 400g), Ware et al. (1990) found that concentrations $\geq$ 0.5 mg/kg in muscle were recorded for those collected from 51 of 80 major lakes and streams. High levels were found throughout the state, but the highest fish

tissue concentrations were reported in the Everglades. In many areas of the southern part of the state, largemouth bass had ≥2.0 mg/kg total mercury concentrations. Lower trophic level species tested in this study, such as the bluegill, generally had tissue mercury concentrations of less than 0.5 mg/kg (except at Bricklake - $\bar{x}$ = 1.01 mg/kg). Bowfin and gar had concentrations similar to the largemouth bass; in bullhead Hg measured $\bar{x}$ = 0.89 mg/kg in the Everglades National Park; oscars had concentrations of $\bar{x}$ = 0.58 mg/kg and warmouth $\bar{x}$ = 0.94 mg/kg in Conservation areas II and III.

The majority of mercury found in fish is in the monomethylmercury form, and while most mercury in the environment is in the inorganic form, there is usually very little inorganic mercury found in fish (Huckabee et al., 1979; Grieb et al., 1990). Methylmercury is absorbed by fish more readily and excreted more slowly than inorganic mercury. Fish can accumulate methylmercury from both food and the water (Spry and Wiener, 1991).

A variety of factors can affect the accumulation and concentration of methylmercury in fish. In the study by Ware et al. (1990) there was a negative association between mercury concentration in fish and the lake trophic status. A negative relationship between water pH and tissue mercury levels has also been observed in acidic lakes which have fish with elevated mercury levels (Spry and Wiener, 1991). Other factors which can affect levels and toxicity of mercury in fish include water temperature, oxygen levels, and concentrations of dissolved calcium (Spry and Wiener, 1991).

A variety of other Florida wildlife species have been tested for mercury levels. Mottled duck, white-tail deer, crayfish, and frogs all had less than 0.25 mg/kg Hg,

softshell turtles had about 0.6 mg/kg Hg, alligators had about 2.9 mg/kg Hg, and Florida panther had 19 mg/kg Hg (Ware et al., 1990). While seven out of 11 panthers in that study had high Hg levels, one which had been found dead of unknown causes in the Everglades also had an unusually high liver Hg of 94 mg/kg (Ware et al., 1990).

In a separate study, Everglades alligators were found to have very high liver and kidney Hg levels of 40±24 and 26±15 ppm wet weight respectively, while non-Everglades alligators had only 2.5±4.4 and 1.6±2.4 ppm Hg in liver and kidney respectively (Heaton-Jones et al., 1997). An interesting finding in the Heaton-Jones study was that in spite of the high liver and kidney Hg levels in Everglades alligators, brain and spinal cord Hg levels were all less than about 2.5 ppm, and on average only about 1.3ppm. In birds with comparable liver Hg levels, brain levels were usually much higher than the brain levels in these alligators (Scheuhammer, 1988; Frederick et al., 1997).

Hepatic, Renal, and Hematologic Systems

Although MeHg can affect the liver and kidney, clinical manifestation of these effects is uncommon. Effects on liver can involve biochemical alterations and possibly pathological changes. Several cases of organic mercury poisoning in humans resulted in renal signs such as polyuria, polydypsia, and albuminuria (ATSDR, 1994). In most cases, however, reports of renal effects are confined to biochemical or histopathological alterations. In avian species there is, likewise, sparse data on hepatic, renal, or hematologic affects of MeHg.

Liver biochemistry effects in great egrets dosed with 5.0 mg/kg dietary MeHg included increased lipid peroxidation, decreased GSH-peroxidase activity, and decreased

total thiol and protein-bound sulfhydryl concentrations (Frederick et al., 1997). Kidney biochemical effects in the same study included decreased protein-bound sulfhydryl concentrations and GSH-peroxidase activity, and increased glutathione concentrations, glucose-6-phosphate dehydrogenase and glutathione transferase activities.

Methylmercury exposure in great egret nestlings also caused decreased PCV, TPP, and lymphocyte numbers. Plasma biochemical values were also altered and included decreased albumin, uric acid, and LDH and increased AST, ALT, and cholesterol (Frederick et al., 1997). In mice methylmercury caused an increase in PCV and no change in white blood cells (Ilback, 1991). In rats MeHg caused no change in CBC values, but caused an increase in BUN and alkaline phosphatase (Verschuuren et al., 1976).

Reproduction and Behavior

In birds there have been reports of reproductive and developmental effects in ducklings from exposed parents. In the study by Heinz and Locke (1976), ducklings from exposed parents had decreased hatching ability, increased embryonic mortality, and overt neurological symptoms such as loss of balance and tremor (depending on exposure level). Lesions in the CNS included the cerebrum, optic lobes, and cerebellum. The investigators proposed that the mercury fed to the ducklings after hatching did not contribute to the toxicity in this study (Heinz and Locke, 1976).

Fimreite (1971) studied the reproductive effects of methylmercury in ring-necked pheasants and found a number of effects on reproductive success including decreases in egg production, egg weights, hatchability, and increases in number of shell-less eggs and embryonic mortality. Another interesting finding was altered color of eggs with MeHg

exposure. The degree to which this type of information can be extrapolated to wild populations is uncertain because of large variations among avian species and their breeding patterns.

Breeding and nesting behavior in adult birds is also an aspect of concern. Barr (1986) studied the nesting success of loons in mercury-contaminated waters in Canada and concluded that there was decreased nesting success in areas where average prey species contamination exceeded 0.4 ppm. Reduced egg laying and nest site territorial fidelity were associated with mean mercury concentrations of 0.3 - 0.4 ppm in prey items and 2-3 ppm in adult loon brains and eggs.

A risk assessment conducted by Jurczyk (1993), based on a lowest observable adverse effect level (LOAEL) for reproductive effects in loons (Scheuhammer, 1991), concluded that great egrets consumed 3.9 times the LOAEL of 0.059 mg/kg/dy, and can be considered at risk for these types of effects. In many of the wading birds of south Florida high liver Hg concentrations have been found (Sundlof et al., 1994), often well above levels which have been associated with egg shell thinning and decreased hatchability (Fimreite, 1971; Heinz, 1976).

Other than behavioral effects relating to embryonic exposure or to reproductive success, few studies have included more generalized behavior patterns. In the study of nestling great egrets, Frederick et al. (1997) found increased righting time, decreased cleanliness, and increased time spent in shade in birds dosed with 5.0 mg/kg dietary MeHg. Additionally, the egrets appeared to spend less time in active, energetic behaviors.

In humans and other mammals, mercury, especially organic forms, can cross the placental barrier and may also have a higher affinity for the fetal CNS than for the adult

CNS. Possible explanations for the higher affinity for the fetal CNS may relate to metabolic differences in the fetus, increased protein synthesis, slow movement of mercury from fetus to mother, or hormonal changes associated with pregnancy (Choi, 1989). The lesions in infants may be more severe than adults depending on the dose, duration, and time of fetal exposure.

Psychomotor retardation in humans is the main result of prenatal MeHg exposure. This can present clinically as a delay in learning to walk and talk and in increased incidence of seizure (Goyer, 1996). The most prominent alterations in the nervous system are abnormal neuronal migration and derangement in the basic structure of the grey matter, including abnormal organization of brain nuclei and layering of neurons (Choi, 1989). The ability of MeHg to interact with DNA, RNA, sulfhydryl groups, and microtubules may be a contributing factor in the abnormal neuronal migration patterns associated with this toxicity (Choi, 1989; Goyer, 1996).

Levels of Mercury in Wild Avian Species

Cormorants from south Florida were found to have high tissue mercury levels (Sepúlveda et al., 1998). Liver, kidney, and brain Hg concentrations ranged from 0.44 - 250, 0.25 - 77, and 0.05 - 8.4 mg/ kg wet weight, respectively. Between 12% and 15% of the cormorants tested had ≥50 mg/kg liver Hg, and 32 % had brain Hg ≥2 mg/kg. In comparison to another almost strictly piscivorous species, osprey liver Hg levels were also found to be elevated in a number of birds. Between 1964 and 1973, 18 ospreys from the eastern United States were sampled and it was found that 8 of the birds had liver Hg < 1.0 ppm, 9 birds had 1.3 - 6.2 ppm, and one bird had 67.5 ppm (Wiemeyer et al., 1980).

Between 1975 and 1982 only 5 of 21 ospreys had liver Hg levels below 1.0 ppm, 12 had 1- 10 ppm, and 4 ospreys had greater than 10ppm (Wiemeyer et al., 1987).

Blood and liver levels reported in Florida bald eagles were relatively low and well below those associated with overt neurotoxicity, although some were within ranges associated with reproductive or behavioral changes in other avian species (Wood et al., 1996). The range of liver Hg for adult eagles was 0.63-12.20 ppm, while in subadults and nestlings, maximum values were 5.42 and 1.01 ppm, respectively (Wood et al., 1996).

Sundlof et al. (1994) reported liver mercury levels for seven species of wading birds in south Florida. Birds, especially great blue herons, from the central Everglades and eastern Florida Bay areas had higher liver mercury levels than other areas. Fledgling and young adult birds had higher average liver mercury (avg. for all species tested having been 1.68 µg/g) than small nestlings (avg. for all species tested having been 0.62 µg/g). Birds whose diet consisted of larger prey items also had higher liver mercury concentrations (Sundlof et al., 1994).

Pathology of Methylmercury

Gross and Histopathology

Gross pathology in avian species is often unremarkable. Some studies have reported muscle atrophy or emaciation, hemorrhage in the brain, and pale kidneys (Pass et al., 1975; Borg et al., 1970; Finley et al., 1979). In birds histological changes at the light-microscopic level are usually limited to the nervous system and include nerve cell degeneration and demyelination in the brainstem, midbrain, cerebellum, and cerebrum

(Pass et al., 1975; Borg et al., 1970; Heinz and Locke, 1976; Fimreite and Karstad, 1971). Axonal swelling and demyelination of peripheral nerves has also been reported (Pass et al., 1975; Borg et al., 1970; Fimreite and Karstad, 1971). Liver, kidney, and muscle may also be affected histologically (Bhatnagar et al., 1982; Pass et al., 1975), but this has been less frequently documented in avian species.

Characterization of neuropathology of MeHg in birds is variable and depends largely on the dose and duration of exposure. Pass et al. (1975) reported axonal degeneration in peripheral nerves, dorsal spinal nerve roots, and proprioceptive tracts and degeneration of neurons in the dorsal root ganglia. Vacuolation and ellipsoid formation in the white matter tracts was also observed between the cerebellum and medulla and the spinocerebellar nuclei. Focal and diffuse microgliosis and astrocytosis were prominent. Changes in the grey matter of the telencephalon included shrunken neurons with pyknotic nuclei or swollen neurons with central chromatolysis in the paleostriatum, dorsal medullary lamina, and the ectostriatum.

Another study in goshawks found focal demyelination of the cerebellar medulla and of the medulla oblongata, degeneration of nerve cells with chromatolysis and shrinkage, and gliosis (Borg et al., 1970). The cerebrum did not appear to be affected. The peripheral nerves had focal myelin degeneration and axonal swelling.

Neuronal pathology in ducklings of MeHg exposed parents exhibited widely disseminated focal area of demyelination, neuron shrinkage, and necrosis (Heinz and Locke, 1976). There was little or no glial reaction; the granular layer of the cerebellum was affected but Purkinje cells appeared normal. Additionally, the optic lobe had foci of demyelination, neuronal swelling, and necrosis.

Little has been reported on the ocular effect of mercury in birds. Disruption of photoreceptor disc lamellae and vacuolation of the nonpigmented ciliary epithelium was noted in an egret exposed experimentally to 5 mg/kg MeHg daily for one month (Loerzel et al., 1996). Additionally, mercury localization in the egrets resulted in granule deposition in the ciliary epithelium and in several retinal layers of the dosed bird. Unfortunately, this was not a well-controlled study since the three other birds in the study (two controls and another dosed bird) died prematurely.

Lesions in the human central and peripheral nervous systems can be widespread. In adult humans the granular cell layer of the cerebellum and the calcarine cortex of the cerebrum seem to be selectively affected by MeHg toxicity (Shaw et.al., 1975). The histological findings correlate with some of the symptoms such as ataxia, constriction of visual fields, and impaired speech (Shaw et al., 1975). In severe cases the brain may be atrophic with neuronal necrosis in the cortex. The granule cells appear to be the most susceptible to MeHg with cells degenerating beneath the Purkinje cell layer (Komulainen, 1988). In extreme cases Purkinje cells may also be lost (Tacheuchi, 1985). The peripheral nervous system is less affected than the CNS in humans. Sensory fibers are more sensitive to MeHg than motor nerves (Komulainen, 1988).

In the fetus, methylmercury causes hypoplasia and underdevelopment of the brain. In the Minamata and Iraqi cases, there was a high degree of similarity in the brain lesions of infants exposed in utero at that time, even though the exposures were different with regard to doses and duration. The primary disturbances in the developing brain were an abnormal neuronal migration and a derangement of the basic structure of the grey matter (Choi, 1989).

Lesions in several experimental animals species studied are not consistent, possibly due to species variation in handling or susceptibility to MeHg or due to variations in dose and route of administration in the various studies. Monkeys are the most similar to humans in that the calcarine cortex is affected and early changes in vision are detected (Berlin et al., 1975). Chronic MeHg toxicity caused lesions in the cerebral cortex lining the calcarine and insular cortices, while in acute toxicity the dentate nucleus of the cerebellum and the lateral geniculate nucleus appear to be more susceptible. The granular cell layer of the cerebellum does not become involved in acute or chronic exposure, as seen in humans (Shaw et al., 1975). The cerebellum appears to be the primary target in cats. Peripheral nerves and nerve roots are the primary targets in rats. Other domestic animals such swine, cattle and dogs also exhibit species variability in the nature and location of gross and microscopic lesions from organic mercury exposure (Thomson, 1988).

Distribution in Neural Tissue

Histochemical and autoradiographic techniques have shown that mercury appears first in glia cells and macrophages (Hargreaves et al., 1985; Berlin et al., 1975), and as symptoms appear, mercury also becomes visible in neurons. In the rat the relative decreasing concentration of Hg in various brain regions is spinal ganglia > cerebral cortex > cerebellum (Somjen et al., 1973). The regional variation in non-human primates may be greater, with the calcarine cortex, corpus striatum, and the lateral geniculate having the highest concentrations (Evans et al., 1977). The calcarine cortex has the highest Hg concentrations in the dog brain, as well (Yoshino et al., 1966b). Subcellulary MeHg has been reported to have a fairly even distribution between mitochondrial, synaptosomal,

microsomal, and supernatant fractions (Yoshino et al., 1966b; Syversen, 1974). Mercury localization using the autometallography silver-enhancement technique results in the concentration of silver granules mainly in the lysosomes of most cell types including those in the CNS (Møller-Madsen, 1991). It is thought that in most species a certain critical level of about 4 - 10 μg Hg/g brain tissue has to be reached to elicit signs of toxicity (Komulainen, 1988), although histological changes are not closely related to content of MeHg in various regions or to histochemically localized mercury (Somjen et al.,1973).

A microautoradiographic study in mallard ducks (Pass et al.,1975) indicated marked concentration of silver grains primarily over the cytoplasm of astrocytes, in neuropil, and around vessels. Grains were most apparent over astrocytes in the ventral horn, the medullary and cerebellar nuclei, cerebellar peduncles, mesencephalic and diencephalic nuclei, and the striatal layers of the hippocampus. There were similar findings in the cerebellar folia, optic lobes and large white matter tracts. No specific pathologic changes were noted in this study, only silver granule localization.

Identification of Mercury in Tissue Specimens

Several techniques for identification of Hg in tissues are available and include several variations of a silver amplification technique (Umeda method), an osmium fixation technique (Silverberg method), and autoradiography with Hg^{203}.

Silver amplification of mercury sulfides and selenides is based on the ability of metal sulfides and metal selinides to catalyze the reduction of silver ions to metallic silver, which form silver granules in tissue section. The photographic emulsion technique (Umeda method) utilizes a developer consisting of citrate buffer, hydroquinone (reducing

agent), silver lactate (silver ion supply), and a gum arabic solution (Danscher and Møller-Madsen, 1985). Following development, the slides are exposed to sodium thiosulfate and then counterstained. The specificity of this technique for mercury compared to other metals is reportedly quite good based on observations that the silver amplified catalyst is resistant to acids, potassium cyanide, and oxidation (Danscher and Møller-Madsen, 1985). This indicates that the amplified accumulations are not sulfides of zinc, copper, lead, silver, or cadmium, and gold can be eliminated if no exposure occurred. This technique is problematic in that granules appear mainly in lysosomes and may represent a nontoxic form of mercury in tissues (mercury sulfide polymers $(HgS^2HgS^2Hg)_n$), and probably not all of the mercury in the tissues is stained (Danscher et al., 1994; Danscher and Møller-Madsen, 1985). Furthermore, in tissues with substantial amounts of melanin clear identification of the silver granules would be difficult.

In the Silverberg method (Danscher and Møller-Madsen, 1985), sections are preseved with osmium, and similar electron-dense granules are found in the lysosomes. Both the osmium method and the photographic emulsion techniques can be modified for electron microscopic visualization. Energy dispersive microanalysis (EDX) can be used to evaluate relative levels of mercury and other elements in tissue at the subcellular level, but this does not actually provide a visualization of the mercury.

Vision in Birds

Adaptations of the Visual System in Birds

While much of the structure and function of the avian eye is similar to mammalian ocular systems, a number of differences and specialized adaptations are apparent. Flight is

the most obvious characteristic that requires special visual and neural adaptations. The wide range of habitats and niches that birds fill is also a source of many differences among the various avian species. In general, birds have very large eyes in proportion to the size of their head. Ostriches have the largest eyes of any land vertebrate, with a diameter of 50mm (Sillman , 1973). In most avian species, the eyes are so large that only a thin common orbital wall separates them (Prince, 1956).

Eye shape in birds is also variable. The majority of birds have "flat" eyes, with relatively compressed anterior-posterior axes (Sillman, 1973). Globular type eyes, which have similar horizontal and vertical axis dimensions, are found in most Falconiformes and some Passeriformes, and tubular-shaped eyes, which possess a relatively elongated vertical axis, are found in most owls (Sillman, 1973). Avian eyes also contain scleral ossicles, which encircle the anterior eye in the area of the limbus and provide support for corneal curvature. Avian eyes also tend to be asymmetrical, especially in the nasal-temporal direction, resulting in an asymmetrical arrangement of the retina around the optical axis (Martin, 1993). The degree of lateral placement of the orbits is also variable among birds and may reflect an adaptation to varied foraging needs. Predatory birds often have more frontally directed eyes for increased depth of field compared to grain-eating species.

The avian eye also differs from mammalian eyes in that the retina is avascular. Instead the avian retina is partially nourished by a pecten, a pleated or folded highly vascular structure, with its base located over the optic disc. The pecten can be quite variable in size and shape among the different avian species (Sillman, 1973).

The cormorant eye is of moderate size compared to other birds. The globe is of the flattened type and, as in most birds, contains scleral ossicles. Cormorants have a relatively

thick and flattened cornea (Sivak et al., 1977). The presence of the flattened cornea may reflect an ecological adaptation for the underwater foraging of these birds. The cornea can function in accommodation processes, and its shape may affect light-refraction characteristics. Cormorants also have highly-developed muscles associated with the iris (Meyer, 1986), which may function in a sphincter-like manner on the lens, as well as perform the usual function of controlling pupillary size. The action of these muscles may alter the shape of the lens and increase the ability of amphibious birds to accommodate underwater. Most diving birds have a larger contribution of lenticular accommodation compared to corneal accommodation (Meyer, 1986).

Visual Pathways and Organization of the Brain

The organization of the nervous system in birds, especially the brain, differs in a number of ways from mammalian species. On a gross structural level avian, reptilian, and mammalian brains have similar patterns consisting of five major subdivisions including the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. They vary, however, in their internal neural organization and pathways, and in the relative proportions of various regions. The avian brain is lissencephalic with almost no convolutions. The internal organization of the avian brain consists of broad fields of cells and nuclear clusters (termed striatum), with only a thin (2-3 cell-layers thick, <1 mm) cerebral cortex (Benowitz, 1980). In contrast, the mammalian brain has a highly developed laminar cortex with many sulci and gyri. Despite the morphological differences, embryological, anatomical, and histochemical studies suggest that most elements of the mammalian and avian telencephalon are comparable (Benowitz, 1980).

An additional difference between the avian and mammalian brain is the development of a very large, laterally placed optic tectum in birds. This modification presumably reflects the importance of vision in birds. The large size and ventrolateral location of the eyes, and the presence of the optic lobe are the main cause of the dorso-caudal shift of the brain in the skull (Benzo, 1986). However, cormorants and others of the pelecaniform genera have somewhat smaller eyes which are located more rostrally than many other avian species and the brain and bill axes are nearly parallel, an orientation comparable to other vertebrates (Pearson, 1972).

Visual impulses from the retina are carried in the optic nerve which decussates almost completely at the optic chiasm. Visual information continues via the optic tract into the brain and is relayed by several routes. The three main systems for visual information processing include the thalamofugal pathway, the tectofugal pathway, and the accessory optic tract (Benzo, 1986; Breazile and Hartwig, 1989). The accessory optic tract terminates in the accessory optic nucleus (nucleus ectomammillaris or the nucleus of the basal optic root) (Benzo, 1986). This nucleus, thought to be a relay in oculomotor function, projects efferent fibers to the oculomotor nuclei, the vestibular nuclei, and the cerebellum (Benzo, 1986).

The majority of visual information in birds, as in mammals and reptiles, is conducted by two major pathways. The thalamofugal pathway projects from the retina directly to the nucleus opticus principalis thalami (OPT) which corresponds to the nucleus geniculatus lateralis pars dorsalis of mammals (Breazile and Hartwig, 1989; Güntürkün et al., 1993). From the OPT fibers are conveyed to the ipsilateral and contralateral visual

Wulst (the hyperstriatal complex in the anterior dorsal aspect of the cerebral hemispheres). This pathway is presumed to correspond to the geniculostriate pathway in mammals (Shimizu and Karten, 1993). The tectofugal pathway projects from the retina to the optic tectum, to the nucleus rotundus in the thalamus, and then to the ectostriatal complex in the telencephalon. This pathway has been compared to the colliculothalamo-cortical pathway in mammals (Shimizu and Karten, 1993). Most birds have laterally placed eyes, and the dominant pathway for visual processing is the tectofugal pathway, but this can be species dependent. In owls, for example, both pathways are well represented, but the thalamofugal path is much more prominent than in pigeons (Bravo and Pettigrew, 1981; Güntürkün et al., 1993).

Electrophysiology

Overview of ERGs and VEPs

Electroretinograms (ERGs) and visual evoked potentials (VEPs) are functional tests which measure the combined responses of numerous cells in the retina and central visual pathways, respectively. The most common methods of recording ERGs and VEPs include the flash ERG (FERG), pattern ERGs (PERG), flicker fusion frequency (FFF), flash VEP (FVEP), and pattern VEP (PVEP). The following discussion will focus primarily on FERGs and FVEPs, since those were the tests included in this study.

The FERG tests the function of the retina by recording, at the cornea, the electrical potential difference generated as a result of stimulation by a single flash of light. The response is a waveform (Figure 2.1) which represents a composite of responses from

various cells of the retina. The ERG is a graded potential which means that the degree of response is directly related to the magnitude of the stimulus. The main components of the FERG that are most often evaluated clinically are the corneal negative a-wave followed by a positive b-wave (Figure 2.1). Other significant components of the FERG that will not be discussed here include oscillatory potentials, slow, long latency c-wave, early receptor potential (ERP), and the scotopic threshold response (STR).

The initial part of the a-wave is thought to be generated by the photoreceptors (rod and cone cells), and the later part is thought to be a composite of photoreceptor, bipolar, and other inner retinal component responses (Robson and Frishman, 1996). The b-wave arises primarily from the interaction of Müller and bipolar cells in the retina (Miller and Dolwing, 1970; Hood and Birch, 1996; Berson, 1981). In birds it has been shown that the maximal amplitudes of the a-wave correspond to the outer portion of the receptor layer, and maximal b-wave amplitudes correspond to the inner nuclear layers, although the exact cellular components contributing to the responses have not been isolated (Ogden and Wylie, 1971).

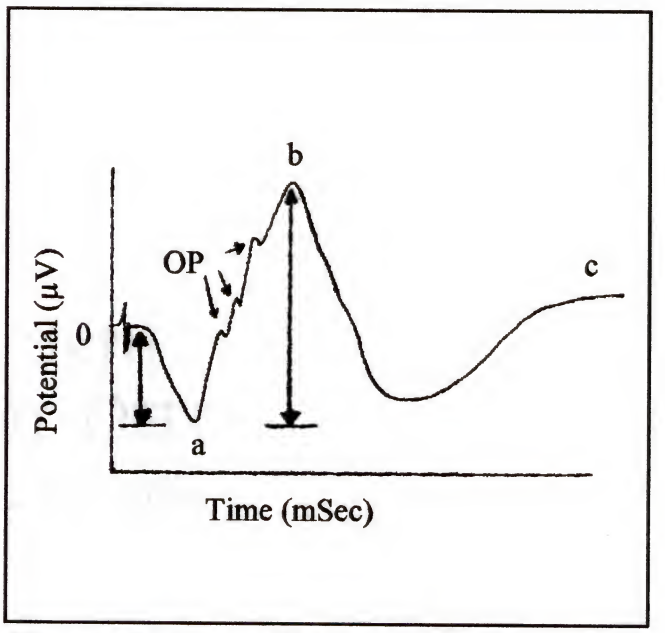


Figure 2.1 : Typical ERG waveform showing a, b, and c waves; oscillatory potentials (OP).

Various disease states involving retinal degeneration or dysfunction can cause alterations in the amplitudes and latencies of the a- and b-waves (Aguirre and Rubin , 1972; Carr and Siegel, 1990; Nilsson et al., 1992). Additionally, the rod and cone function can be separated with various ERG techniques, enabling detection of disease states that preferentially affect one of these cell types (Acland, 1988). Electroretinograms are used to evaluate retinal function in many species and have been used extensively to evaluate retinal function in cataract patients prior to surgery.

Visual evoked potentials (VEPs), which test occipital cortex function and evaluate the integrity of the postretinal visual pathways, have been used in the examination of MeHg toxicity (see below). VEPs have been used in humans and a variety of animals for the study of disease, toxicology, and physiology (Strain et al., 1990; de Weerd, 1990). The VEP waveform is somewhat variable among species (Creel et al., 1973), but basically consists of a series of positive (P) and negative (N) deflections that are usually labeled in series according to the order that they occur (P1, N1, P2, etc.) or according to their latency (e.g., P15, N100). Latencies of peaks can be measured from the onset of the stimulus. VEP amplitudes can be measured either from the baseline or from peak-to-peak with respect to the nearest neighboring component. The peak-to-peak method is probably the most frequently used, and it tends to provide more reproducible and reliable results than the baseline measurement method (de Weerd and Stegeman, 1990).

Naka-Rushton Equation

In addition to evaluating ERG a- and b-wave amplitudes and latencies individually, a mathematical model can be used to describe the intensity-response profiles. The

modeling approach can be used for any of the ERG parameters, but the most common application of this technique has been the characterization of the stimulus intensity versus b-wave amplitude relationship using the Naka-Rushton equation.

The Naka-Rushton equation,

$$R(I) = \frac{R_{max} \times I^n}{I^n + K^n},$$

is a nonlinear, mixed-effects regression where R(I) is the b-wave amplitude at stimulus luminance I, R(max) is the theoretical maximum response amplitude, K is the stimulus intensity at which half-saturation occurs, and n is a dimensionless constant indicative of the slope of the function.

This model has been used to evaluate retinal function, especially with regard to hereditary and degenerative retinal diseases (Dagnelie and Massof, 1993; Gangadhar et al., 1989). The parameters R_{max}, log K, and n have been shown to reflect functional aspects of the retina. R_{max} represents the number of active photoreceptors of the retina; the log K is an indication of the retinal sensitivity, and n represents the number of simultaneously responding photoreceptors (Anastai et al., 1993; Hood et al., 1994). A loss of the capacity of the retina to respond would cause a decrease in the R_{max}, and a loss of retinal sensitivity would result in a shift of the log K value to the right.

Factors Affecting ERGs and VEPs

Ocular electrophysiology results can be affected by a variety of confounding factors which need to be controlled for during testing by establishing standardized

laboratory procedures. Factors influencing ERGs and VEPs include stimulus intensity and wavelength, pupil size, level of light or dark adaptation, level of anesthesia, intraocular pressure, temperature, oxygen and glucose status of the animal, and age (Komaromy et al., 1998). Differences in proportion of rods versus cones, density and distribution of photoreceptors, and variations in neural pathways may lead to species-specific characteristics in ERGs and VEPs. Visual evoked potentials may also vary greatly depending on electrode placement. Bioelectrical artifacts may also interfere with evoked potential recording and can include EEG, EKG, eye blinks, eye movements and muscle activity (de Weerd and Stegeman, 1990). In VEP recordings of dogs, artifacts from ERGs were also considered (Strain et al., 1990).

Use of Ocular Electrophysiology in Toxicology

Electroretinograms and VEPs, as well as other electrophysiological tests, have been used to evaluate human and animal exposure to various toxicants , including mercury (Tokuomi et al., 1982; Somjen et al., 1973; Dési et al., 1996; Lamm and Pratt, 1985; Mattsson et al., 1981; Gramoni, 1980; Gitter et al., 1988), lead (Tessier-Lavigne et al., 1985; Sillman et al., 1984; Otto and Fox, 1993), organophosphates (Yoshikawa et al., 1990), and many other substances (Araki and Murata, 1993; Rebert, 1983; Weisse et al., 1995). A large number of compounds can produce sensory effects, and of those, many also can produce adverse effects on the visual system (Geller er al., 1995). Ocular electrophysiological tests are useful in characterizing harmful effects of neurotoxicants on the visual system, because they provide a noninvasive means of assessment on a functional

level and can facilitate interspecies comparisons. The various tests are also valuable in that each can be used to evaluate a different part of the visual pathway, and in combination they can provide complementary functional assessment of the integrity of the visual system.

Effects of Mercurials on the Visual System

Visual effects are usually an early sign of methylmercury toxicity, and the most striking complaint is a very symmetrical visual field constriction. Much of the initial vision data was generated from the human exposures in Japan and Iraq, while research has further characterized these effects and attempted to understand it mechanistically. A study of macaque monkeys treated with methylmercury resulted in constricted visual fields, as well as decreased flicker sensitivity and flicker resolution (Merigan et al., 1983). Other visual disturbances ranged from blurred vision to blindness (Sabelaish and Hilmi, 1976). It generally has been considered that the visual deficits resulting from mercury toxicity are due to central nervous system damage following the accumulation of mercury in visual cortical areas. Some studies, which have shown altered ERGs or VEPs, may also indicate possible involvement of the optic nerve or retina in this syndrome.

Rats exposed to 0.5 mg/kg daily methylmercury chloride subcutaneously for 9 weeks had changes in ERG patterns, high ocular mercury levels and electron-dense granules of mercury deposited in the retina (Gramoni, 1980). This study resulted in longer a-wave latencies than found in controls, and a- and b-wave amplitudes that were greater until the fourth week and smaller after the fifth week compared to that of controls. The concentration of mercury in the eye reached 5.4 ± 1.2 µg/g, while that of the brain had 4.2

± 0.8 µg/g (Gramoni, 1980). Gitter et al. (1988) also found increased scotopic b-wave amplitudes in cats dosed with mercury acetate at doses of 0.025 - 0.25 mg/kg/day for 2.5 - 4 weeks.

The cause of the increased amplitudes is uncertain, but it has been speculated that perhaps it is related to enhanced axonal transport in the optic system of the rat after exposure to MeHg (Gitter et al., 1988). Evaluation of workers chronically exposed to inorganic Hg supported the use of evoked potentials as a sensitive detector of subclinical neurologic damage (Lamm and Pratt, 1985). This study found that nerve conduction times were delayed peripherally, but accelerated from the brain stem to the cortex, and that these were detectable subclinically. It was suggested that perhaps the shortened latencies for cortical and subcortical potentials may be explained by damage to inhibitory mechanisms in the CNS by the binding of mercury (Lamm and Pratt, 1985).

A preliminary study conducted on Great Egrets (Loerzel et al., 1996) resulted in decreased ERG scotopic b-wave amplitude and increased b-wave latency in one bird dosed with about 5.0 mg/kg methyl mercury subcutaneously for four weeks. The egret study, however, was not well controlled, and the bird was an adult from a wildlife center, with an unknown history.

In vitro studies have also shown that mercury can affect ERG parameters. Isolated bullfrog retinae exposed to Hg^{2+} caused a transient increase in rod, but not cone, receptor potential followed by an irreversible decrease (Fox and Sillman, 1979). Isolated retinae from larval axolotls also resulted in an initial increase in peak voltage response of rods at concentrations of 2.5 to 100 µM $HgCl_2$ for 20 minutes, while longer exposure resulted in

decreased amplitudes (Tessier-Lavigne et al., 1985). In the latter study, no effect was seen on cone responses, and no rod changes were noted at a concentration of 1 μM $HgCl_2$. In both the bullfrog and axolotl studies, the effects of mercury on retinal responses were irreversible.

VEPs in dogs exposed to methylmercury chloride also exhibited decreased amplitudes (Mattsson et al., 1981). Specifically, the dogs had a distortion of the VEP after 1 week which stabilized until 5 -8 weeks and deteriorated rapidly thereafter. In experimental methylmercury poisoning in rabbits, VEPs showed a reduction in amplitude (Iwata, 1980). Visual electrocorticograms in rats dosed with $HgCl_2$ had dose related decreasing tendencies which became significant only after 12 weeks at a dose of 1.6 mg/kg (Dési et al., 1996). In the same study responses from somatosensory and auditory centers were also affected.

Mechanisms of Ocular Toxicity

Methylmercury, due to diffuse distribution and binding in many tissues, has the potential to affect target organs via multiple mechanisms. As discussed earlier, there has been much investigation into the mechanistic aspects of mercury toxicity, and a wide variety of cellular and subcellular components have been shown to be affected. Regarding the ocular toxicity of methylmercury, many of the same mechanisms may also apply.

Vision is driven by the photochemical reaction that is initiated by absorption of a photon by rhodopsin in the photoreceptor disc membranes. This process is linked by a series of biochemical reactions to cation channel closure in the plasma membranes of the outer photoreceptor segments (transduction). Closure of ion channels leads to graded

hyperpolarizations of the photoreceptors, creating an electrical response that is transmitted to the synaptic end of the photoreceptors and then passed on to neurons in the inner retina (Saari, 1992). The ion channels in the plasma membrane are open when cGMP is bound. Following activation of rhodopsin by light, a cascade of reactions results in hydrolysis of cGMP by phosphodiesterase, which lowers cGMP concentrations and allows more channels to close (Saari, 1992).

A study comparing lead and mercury to the phosphodiesterase inhibitor paperivine found that $HgCl_2$ caused transient increases in voltage response followed by an irreversible decrease (Tessier-Lavigne et al., 1985). It was concluded that mercury caused a retinal response consistent with a phosphodiesterase inhibitor since paperivine caused a similar response with a transient amplitude increase followed by a decrease. Since mercury also caused swelling of rod outer segments and rod destruction, it was thought an additional mechanism, such as activity of the sodium pump, which could alter osmotic balance, may be involved (Tessier-Lavigne et al., 1985). Other sources have also described inhibition of ion channels by mercury (Sirois and Atchison, 1996; Leonhardt et al., 1996).

The retina is extremely metabolically active, with very high oxygen consumption rates, and thus could potentially be sensitive to factors affecting these processes (see the section on Biotransformation, Metabolism, and Mechanisms). Additionally, a variety of neurotransmission-related mechanisms could also be a factor in the retinal effects of mercury. Monoaminergic, cholinergic, and GABAergic systems can all be affected, usually exhibited as decreased levels of transmitters or their metabolizing enzymes (Komulainen, 1988). Protein synthesis and axonal transport in the optic system may also be affected by systemic administration of MeHg (Aschner et al., 1987). Other ocular

systems, and the visual pathways and processing, may similarly have the potential to be affected by a variety of mechanisms mediated by mercury.

Other Ocular Toxicants

A large variety of compounds can be toxic to ocular tissues (Millechamp, 1992; Grant, 1986; Weisse et al., 1995). With respect to free ranging wildlife, however, the main risks will be restricted to environmental contaminants. Lead has been shown to alter components of the ERG, as well as the VEP waveforms (Tessier-Lavigne et al., 1985; Fox and Sillman, 1979; Sillman et al., 1984; Otto and Fox, 1993). In most studies the effect of lead was reversible when exposure had ended. Cadmium (Fox and Sillman, 1979), and organophosphates (Yoshikawa et al., 1990) have also caused altered ERG responses.

Arsenic, especially organic arsenicals, can also produce a neurotoxicity similar to organic mercury. Organic arsenicals have been used as feed additives in pigs and poultry, and intoxication has most frequently occurred as a result of excessive dosing of feed. Arsanilic acid, in particular, has been associated with blindness, causing optic disc atrophy and lesions in the optic nerves (Witzel et al.,1976). In swine poisoned with arsanilic acid to the point of blindness, ERG responses remained unchanged, but no VEP response could be elicited in the same animals (Witzel et al.,1976).

The main difference between methylmercury and other toxicants which can affect the visual system, is that methylmercury bioaccumulates in the natural food sources. Thus, for fish-eating birds, methylmercury poses chronic exposure risk which cannot be avoided. Additionally, the neurotoxic effects of MeHg are irreversible, and animals may develop toxicity even after the exposure is removed.

CHAPTER 3
GENERAL METHODS: COLLECTION, MAINTENANCE, AND TREATMENT OF CORMORANTS

Rationale for Species Selection

The double-crested cormorant (*Phalacrocorax auritus*), a species of diving bird in the pelican family, subsists mainly on fish an other aquatic organisms. As a predatory species, cormorants may potentially be exposed to high levels of MeHg due to the tendency of this toxicant to bioaccumulate up the food chain. Few controlled experimental studies on the effects of MeHg have been conducted on aquatic avian species. In order to assess more effectively the environmental risks to wildlife, it is essential to provide reliable baseline information pertinent to the environment and to the species which may be affected. The cormorant is an abundant, wide-ranging species of bird which, due to its life habits, is highly appropriate as a model for the study of MeHg exposure in an avian species.

The selection of birds, in general, as test subjects was supported for a variety of reasons. Since birds tend to mask signs of illness until they become critically decompensated, it is important to devise techniques to detect subclinical indications of exposure. Additionally, methylmercury is known to affect the visual system in some species early in the course of the toxicity (Davies and Nielsen, 1977; Davies et al., 1976;

Sabelaish and Hilmi, 1976). Although birds are highly visual animals, this aspect of MeHg exposure has not been well studied in avian species.

Housing

Facilities for the housing of the cormorants were provided courtesy of the USDA Denver Wildlife Research Center, Gainesville (FL) Field Station. Five 10' x 10' pens were constructed in an open outdoor pavilion with concrete flooring and drainage troughs. Pens were constructed from pre-made panels of chicken wire which were linked together. Each enclosure was prepared by lining the floors with astroturf, various sizes and shapes of natural wood perches, a wading pool, and "nest" baskets lined with twigs and other plant material for the younger birds. Natural perching surfaces were provided to prevent lesions from developing on feet and feathers. For the first 2-3 weeks, the wire panels separating adjacent pens were covered with plastic sheeting from the floor to a height of approximately 4' in order to reduce contact between birds from different pens, to reduce the spread of fecal material between pens, and to provide a calming effect. When the birds acclimated and began perching on higher perches the plastic sheeting was removed.

Cleanliness and hygiene were of great concern during the project. Aquatic birds kept in captivity are notorious for contracting or succumbing to aspergillosis (DeLaRonde and Greichus, 1972). A rigorous cleaning regimen was required to deal adequately with the voluminous, watery feces which cormorants produce. All pen materials, including wading pools, were rinsed twice daily, and nesting material was changed daily until it was removed at the end of the third week. Astroturf was disinfected twice weekly.

Colony Location and Collection Methods

Twenty-six nestling double-crested cormorants were collected on May 23, 1996, under permits FGFWFC # WX95131 and FWS # 804714. They were collected from nesting colonies on Mullet Key and Sandy Hook, small islands (Suncoast Keys) just south of Crystal River in the Gulf of Mexico. The Cooperative Research Unit of the U.S. Fish and Wildlife Service (Gainesville, FL) provided the boat and trailer for initial scouting trips in various locations on the east and west coasts of Florida, as well as for the actual collection of the birds. The Florida Game and Fresh Water Fish Commission (Wildlife Research Laboratory, Gainesville, FL) provided assistance in locating a suitable colony site and also assisted with collection of the birds.

The nesting colony consisted of roughly 75-100 breeding pairs, primarily double-crested cormorants with scattered pairs of brown pelicans, egrets, and herons. The nests were arranged very densely in low mangrove bushes and were constructed of a platform-like, thick layer of loosely woven twigs, sticks, and other plant material. Most nests had two to three nestlings of approximately 2 to 4 weeks of age.

Birds were collected in the early morning and transported in temporary holding cages to the USDA Denver Wildlife Research Center, Gainesville Field Station. The main concerns during the transfer process were hyperthermia and suffocation, since the birds tended to pile up on each other. The risk of losing birds was reduced by using an air-conditioned vehicle, several cages with fewer birds in each one, and by covering the cages with a light cloth to calm them. No birds were lost during collection or transport.

On arrival at the facilities, the birds were separated randomly into pens, five in each, with a sixth bird in the first pen. The birds were force fed for the first few days by

which time all were eating on their own. One of the youngest birds died during the first night, but other than its small size, gross necropsy findings were unremarkable. Notes on other deaths and miscellaneous health concerns can be found at the end of this chapter.

After the first 24 hours, during which the chicks were allowed to rest and were not handled, each bird was given a thorough physical exam, weighed, and leg banded. Identification consisted of number and color-coded leg bands placed on both legs for easy recognition regardless of their position in the pen. An improvised system of leg bands, using plastic cable ties and color tape labeled with indelible markers, was devised since standard poultry leg bands did not fit cormorant legs.

Diet

The base diet for the cormorants consisted of thawed Spanish sardines obtained from Northwest Seafood (Gainesville, FL). Other types of fresh and thawed seafood (squid, shrimp, capelin, and silversides) as well as a vitamin supplement (Purina Mazuri®Vita-Zu® Bird Tablet) were also fed to the birds three times weekly. The birds were fed twice daily with the number of sardines that each individual ate recorded. Daily consumption in grams was calculated using a current average weight for the sardines or other food items (Chapter 4).

Dosing and Blinding Methods

Solutions of reagent-grade methylmercury chloride in acetone were prepared by the Florida Department of Environmental Protection in doses of 1.04 mg/ml and 10.00 mg/ml. Levels of 0.5 and 3.5 mg MeHg/kg daily diet were the low and high dose

treatments, respectively. To blind the study, solutions were needed in concentrations that matched the dosages which were to be administered (i.e., seven-fold difference), such that the same volume would be used regardless of the solution or the dose. The three solutions used for dosing were an acetone blank for controls, 1.04 mg/ml, and a 7.0 mg/ml solution (diluted from the 10 mg/ml solution into acetone).

Dosing was double-blinded such that neither the person dosing nor the persons evaluating the outcomes knew which birds were in each treatment group. The three solutions were each placed in identical tinted glass bottles with appropriate concentration labels. One person randomly assigned numbers 1, 2, and 3 to the solutions and relabled the containers. The solutions were then given to a second person who randomly assigned them with the labels A, B, and C. These steps were performed independently of each other and blinding keys were kept confidentially in separate locations by each individual.

Blinding was maintained throughout the testing and analysis of the ERG data. When the first tissue samples were analyzed for mercury the group assignments became apparent and the experiment was unblinded (Table 3.1). Group A was control, C was the low-dose group, and B was the high-dose group. To maintain objectivity in the assessment of blood and histopathology, slides were covered and re-labeled by a neutral person.

Table 3.1 : Treatment group labels, dosages, and solution concentrations revealed when the study was unblinded. The dose for controls represents background mercury levels.

Treatment Group	Daily dietary dose of MeHgCl mg/kg fish	Solution concentration mg/ml
A - Control	(~0.0237)	0.0
B - High	3.5	7.00
C - Low	0.5	1.04

The cormorant chicks were randomly assigned to one of the three treatment groups: A, B, or C. Determining the sex of the birds prior to assignment to treatments was not possible. Unfortunately, the random assignments resulted in unbalanced female to male ratios, which limited somewhat the analysis of gender-related effects, especially for group B. Group A (control), B (high dose), and C(low dose) had female to male ratios of 2:5, 7:1, and 3:4 respectively.

Birds were dosed once daily based on a daily average adjusted food intake. Averages were recalculated each week to account for changes in their intake. It was decided to use an average daily intake based on a weekly intake for several reasons. First, testing or excessive handling of a bird on any one day often stressed it and considerably reduced intake. Second, birds sometimes became quite stressed for a variety of other reasons (lawn mowers, loud noises, or other activities) and would regurgitate whole or partially digested fish. Generally, these types of disturbances were minimal, however certain events were not controllable. Third, the sardines which were fed as the base diet were fairly large fish, 60-80 grams each, and a change in consumption by just one fish altered the individual daily intake dramatically. As a result of all these factors, it was considered more realistic to use an average daily consumption for the calculation of mercury doses rather than the actual daily intake.

An additional consideration in calculation of food intake was the fact that the birds would regurgitate round masses of scales, fins, and bones on a regular basis. Roughly 5 to 6 of these were found in each pen each day, and they weighed 20-40 grams each. It was rare to actually see the birds do this, but the presence and number of these masses in all pens (each with 5 birds) seemed to suggest that all the birds were doing this. The factors

mentioned above led to an inaccurate picture of intake patterns, especially with regard to the methylmercury to be administered. It was felt that calculations based on total intake would over-estimate actual intake levels and that this should be accounted for. In an attempt to adjust for these variables, an arbitrary value of 10% of the average (20-40 g was roughly 5-15% of intake) was subtracted and the adjusted value was used for the methylmercury dosing calculation. To determine the amount of solution to use in capsules, the 7-day adjusted average intake (kg) was multiplied by 0.5 (ml/kg fish). The 0.5 ml/kg of fish result from dividing either of the desired dietary doses (0.5 or 3.5 mg MeHg/kg diet) by the corresponding solution concentration (1 or 7 mg MeHg/ml). Mercury doses were recalculated in this manner weekly.

Solutions were pipetted into gelatin capsules (Torpac®, Fairfield, NJ), and the acetone was evaporated under a fume hood for 15 to 30 minutes. The small 000 gauge capsules were closed and then inserted into two additional layers of gel caps, 00 and 0 sizes respectively. It was determined that a single layer of gelatine dissolved and allowed fluid to enter in 4 - 5 minutes. The time span between placing the capsules in the fish to the time it was fed to the birds sometimes exceeded 5 minutes. Thus, the triple encapsulation was selected, allowing a sufficient lag period (approximately 15 minutes) that would prevent premature leaking of mercury.

Capsules for each bird were stored in weekly batches in sealed plastic color-coded containers labeled with bird ID and treatment. The dosed capsule was administered in the first fish of the evening feeding. Following feeding, the birds were observed for at least 30 to 45 minutes to ensure that they did not regurgitate the dosed fish. The evening feeding was selected for treatment because during the day they were much more likely to be

stressed and regurgitate their morning food. This system of feeding the capsules in the fish worked well, was minimally stressful on the birds, and was less time consuming than catching each bird and force feeding the capsules individually.

Methylmercury Intake

When the study was unblinded, the specific amount of MeHg administered to each bird was calculated based on assignment of control to group A, low dose (0.5 mg/kg) to group C, and high dose (3.5 mg/kg) to group B. Contribution to the mercury burden, due to background levels of total mercury (inorganic and organic) in the diet, was assessed and factored in as a portion of the exposure. Total mercury in fish tissue was determined by cold vapor atomic absorption (Chapter 5).

The average total MeHg administered, daily total intake ranges, and per-body weight dosages, including background levels in fish, are presented in Table 3.2. The cumulative methylmercury intake is shown in Figure 3.1. The background level of total mercury was 0.0237 and 0.031 mg/kg in sardines and silversides, respectively. All birds were exposed to ≤0.35 mg total Hg from the base diet over the 5 weeks prior to dosing.

Table 3.2 : Mercury intake in cormorants, including background fish levels, expressed in different forms. Numbers in parentheses are 1SD. Daily totals and body weight (b.w.) dosages are expressed as average ranges for 8 weeks of dosing.

Treament/ exposure level (mg/kg diet/day)	Total MeHg administered (mg)	Daily total (mg/day)	Per body weight dose (mg/kg b.w./day)
A (~0.0237)	0.455 (±0.050)	0.006 - 0.009	0.004 - 0.006
B (3.5)	50.000 (±3.286)	0.806 - 0.966	0.587 - 0.741
C (0.5)	8.423 (±0.978)	0.124 - 0.182	0.082 - 0.127

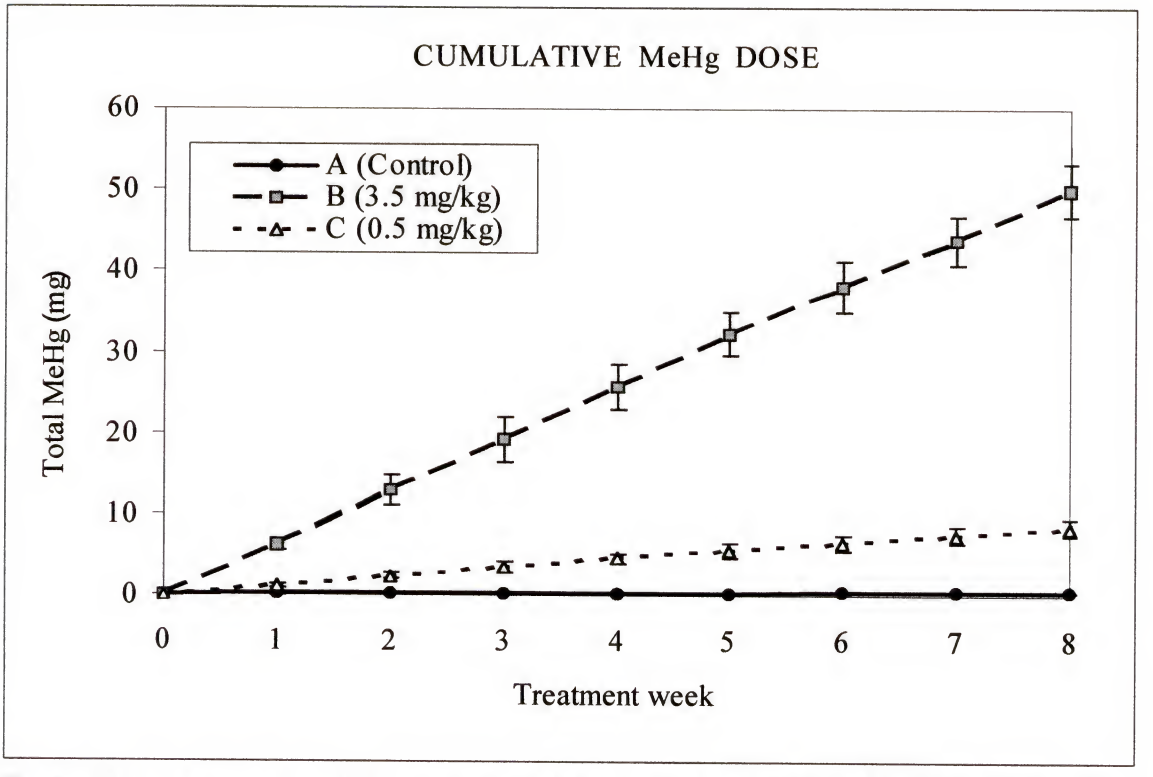


Figure 3.1 : Average, ± 1SD, cumulative MeHg (mg) administered to cormorants over an 8-week dosing trial. Levels adjusted for background Hg in fish.

Statistics

Sets of response variables for each of the subcomponents of this project were evaluated for significant differences due to time, treatment, or time by treatment interactions. For most variables, one-way repeated measures ANOVA and Tukey's multiple pairwise comparisons were used (PROC GLM, SAS 6.1), and the significance level was set at $p \leq 0.05$. The above procedures were applied to multiple response variables including weekly body weights, food consumed, CBC and serum chemistry parameters, and intraocular pressures. Data which was in the form of ratios, such as consumption per body weight and organ weight per b.w., were arc sine square root transformed prior to analysis.

The same analysis was also performed on log-transformed blood, tissue, and fecal mercury levels. Linear correlations between blood, tissue, and fecal-urate mercury levels were also evaluated.

Data from neurological exams was analyzed for within-group change from baseline using the Friedman Rank Sum test, followed by the Wilcoxon Signed Rank test. Between-group comparisons of changes were evaluated using the Kruskal-Wallis and the Wilcoxon Rank Sum tests. Behavioral data, reported as porportions, were arc sine square root transformed and analyzed with PROC GLM as described above.

In general, most of the electrophysiological test parameters, including ERG a- and b-wave amplitudes and latencies, as well as VEP N and P wave amplitudes and latencies, were evaluated in the manner described above for other continuous variables. In addition, the ERG b-wave intensity response profile was characterized by the Naka-Rushton equation, and the parameters of this equation were subsequently compared using the repeated measures ANOVA. Specifics of the fitting of the Naka-Rushton model will be discussed in detail in chapter 7.

CHAPTER 4
GROWTH CURVES, FOOD CONSUMPTION, AND GENERAL HEALTH

Introduction

As a part of the general health assessment of the cormorants, food consumption and weight gain patterns were evaluated. Decreased food intake, loss of weight, or decreased weight gain in young birds can be early signs of illness. In addition to evaluating the potential effects of methylmercury exposure on these indices, these parameters can be useful tools in monitoring the general health of the birds.

Methods

Birds were randomly assigned to treatment group A, B, and C as described previously. The three solutions of methylmercury chloride in acetone were 0, 1 and 7 mg/ml. A volume of solution was administered daily, in gel capsules, to each bird at a level equivalent to 0, 0.5, or 3.5 mg MeHg/ kg diet. All personnel were blinded as to which solution was assigned to the lettered groups. For specifics on the blinding procedure, calculations of dosages, and use of gel capsules, see Chapter 3.

The cormorants were fed a sardine-based diet twice daily as described in Chapter 3. The number of fish or other foods that each bird consumed was recorded on a daily feed form. Attempts to record exact weights of individual fish eaten by each bird proved

impractical; thus, a close estimate of food intake was calculated based on an average weight of 50-100 sardines. Sardine size was quite uniform but a new average weight was calculated for each new shipment that was delivered. Likewise, average weights for the other seafood items were calculated and individual consumption recorded. Intake rates were evaluated based on daily averages for each treatment week.

Each bird was weighed as part of the general weekly physical exam. Body weights to the nearest 2 grams were recorded for several weeks prior to treatment and at the end of each treatment week during dosing. There are no reliable distinguishing characteristics between male and female juvenile double-crested cormorants; thus, assignment to groups balanced according to gender was not possible. The gender of each individual was verified only at necropsy (Chapter 6), and subsequent adjustments in the analyses were included to evaluate for gender-related differences in results. Repeated measures ANOVA and Tukey's multiple pair-wise comparisons were used to compare growth curves and consumption rates during the treatment time.

Weekly physical exams, as well as daily observations of the birds at feeding times, were conducted to monitor any changes in their general health. Any variations in health status were recorded, and conditions requiring medical attention, but which were unrelated to the dosing, were treated in an appropriate manner. Chapter 6 (pathology and necropsy findings) includes further details regarding selected conditions in specific birds.

Table 4.1 : Average daily food consumption (g) ± 1SD. A, B, and C are controls, high-dose, and low-dose MeHg respectively. **Start of dosing.

Week #	A	B	C
-5	399 (± 75)	384 (± 32)	425 (± 49)
-4	418 (± 73)	365 (± 55)	426 (± 36)
-3	447 (± 58)	366 (± 61)	362 (± 72)
-2	406 (± 44)	372 (± 51)	338 (± 46)
-1	389 (± 45)	353 (± 51)	326 (± 64)
**0	327 (± 46)	282 (± 31)	315 (± 50)
1	385 (± 64)	309 (± 54)	378 (± 83)
2	307 (± 60)	292 (± 46)	310 (± 67)
3	301 (± 45)	294 (± 48)	313 (± 81)
4	309 (± 65)	299 (± 35)	309 (± 56)
5	274 (± 45)	268 (± 44)	256 (± 40)
6	268 (± 58)	259 (± 61)	309 (± 53)
7	278 (± 38)	289 (± 27)	285 (± 40)
8	295 (± 50)	290 (± 47)	228 (± 70)

Results

Food Consumption and Body Weights

When the cormorants were initially brought into captivity, around 2-4 weeks of age, they consumed roughly 400 g of fish per day. When offered other types of seafood such as shrimp, squid, or other types of fish, the birds adjusted their intake of sardines accordingly, keeping intake levels constant. The varied seafood offered to the birds comprised only about 5 % of the total amount that they consumed. Over several weeks of acclimation, consumption gradually plateaued around 250-300 g/day (Table 4.1). Figure 4.1 shows the percent body weight consumption, which declined from 30 - 35 % to 15 - 20 % over the 14-week span. There were no significant differences in weights between groups at any time from the beginning of dosing to the end. Group C showed slightly lower consumption, compared to groups A and B, during the last week of dosing, but this was not a significant difference among the groups.

Body weights increased from 1169 g (±190, 1SD) to 1518 g (±141) in group A, from 1112 g (±94) to 1390 g (±119) in group B, and from 1275 g (±91) to 1435 g (±145) in group C. Group B had average weights that were consistently lower, by about 150 g, than the controls and low-dose groups during the entire study (Figure 4.2). Week 8 weight values for group C females were lower than B females ($p=0.0074$), but there were no differences, when adjusted for gender, between controls and treated groups at any time. The disproportionate number of females in group B was the main contributing factor to the differences (Figures 4.2 and 4.3). The slight decrease noted in group C weights during the last week corresponded to the time frame of decreased food consumption.

Physical Exams and Miscellaneous Health Issues

Of the original 26 birds collected 22 were included in the study. All 22 of the individuals which were included survived to the end of the study. Of the four birds that died, one died the first night of undetermined causes, and two developed respiratory illness and died within 2 weeks after capture. The fourth bird, while recovering from anesthesia, fell from the table, sustaining a severe back injury, and was euthanized.

The majority of the cormorants remained quite healthy for the duration of the experiment. Exceptions included varied injuries or illnesses in birds Y-01, O-12, O-17 and G-24. Bird Y-01 was not clinically ill, but on necropsy large encapsulated intrathoracic fungal granulomas, presumably aspergillosis, were seen. Given the size and distribution of the fungal masses throughout the thoracic cavity, it was surprising how well the bird had been doing.

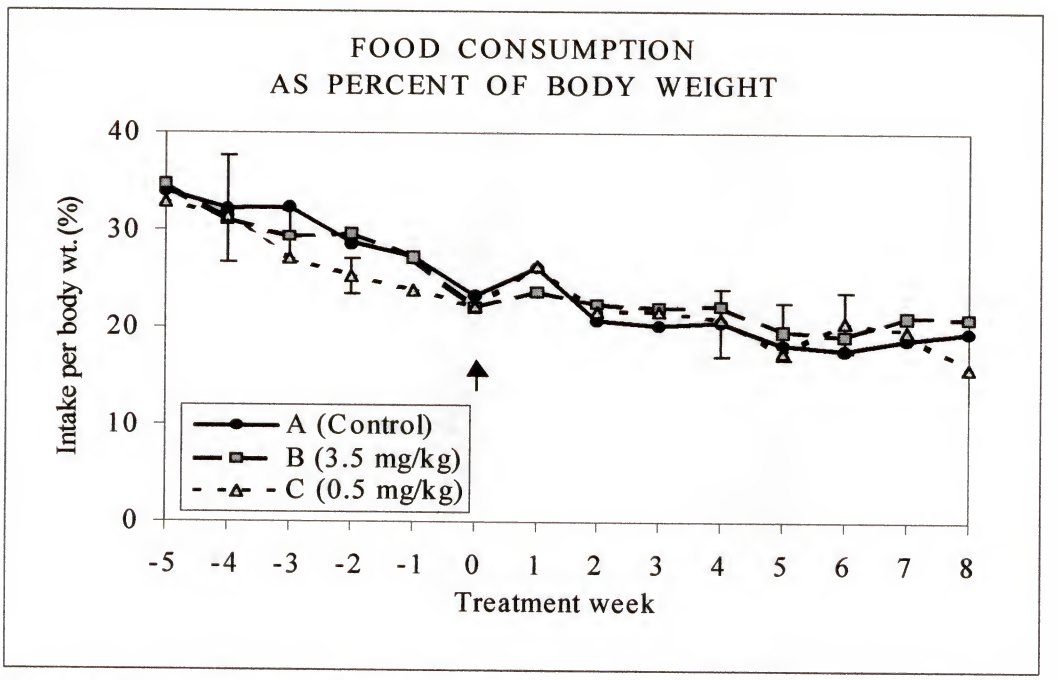


Figure 4.1 : Average daily food intake (based on 7 day averages) as percent of body weight for three treatment groups. Bars are ± 1 SD, and arrow marks start of MeHg dosing.

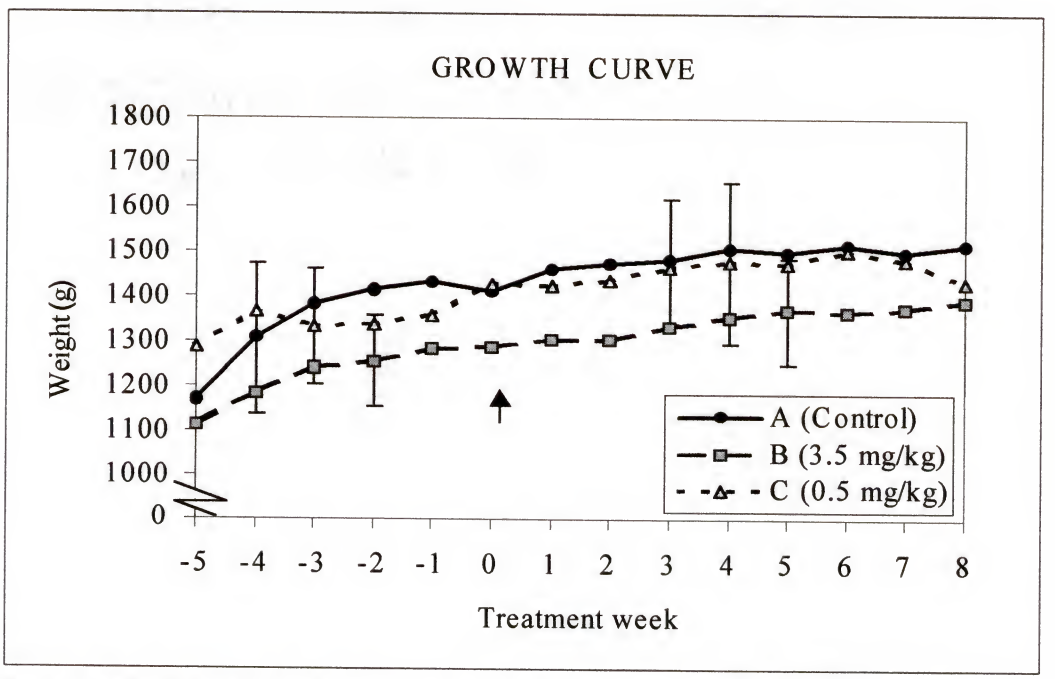


Figure 4.2 : Weekly body weights (g) for three treatment groups. Bars are ±1SD, and arrow indicates start of MeHg dosing.

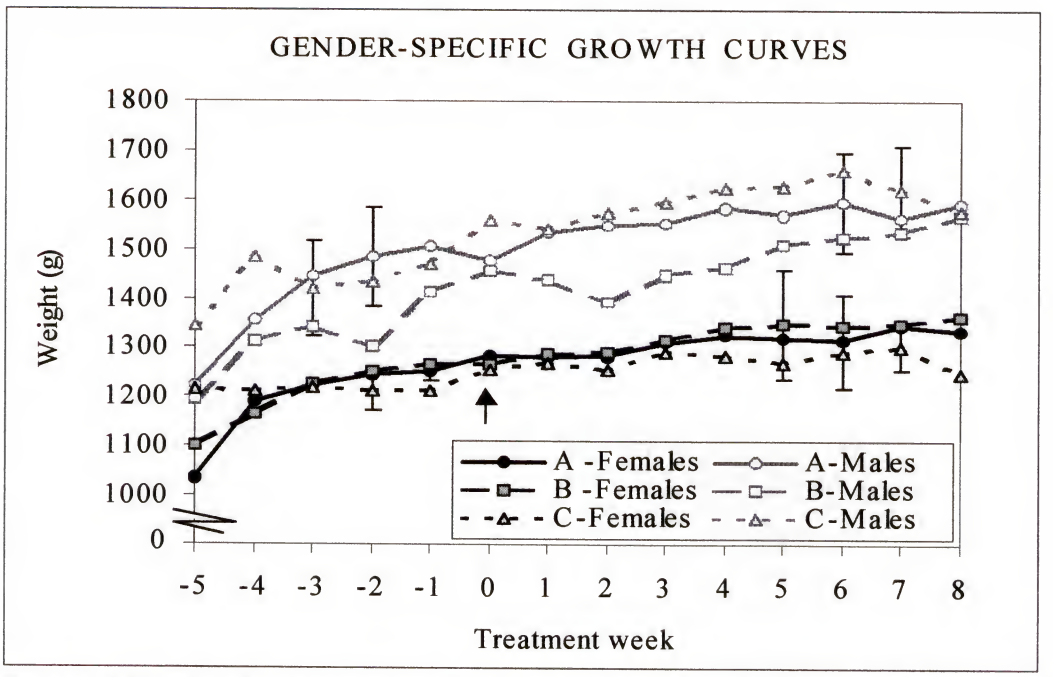


Figure 4.3 : Weekly body weight (g) for females and males within each treatment group. Bars are ± 1 SD, arrow marks start of MeHg dosing. Female-to-male ratios are 2:5 (A), 7:1 (B), and 3:4 (C).

Bird # G-24 consistently became extremely distressed, and struggled when caught or restrained. During one handling session G-24 caused a self-inflicted, 5-cm long, deep neck laceration with his toe nail. Treatment consisted of surgical repair and one week of antibiotics. G-24 recovered without incident and was subsequently much less agitated.

As part of the developing social structure in each pen, aggression was observed but was generally not a serious problem. However, following attacks by R-13, O-12 developed an acutely swollen and inflamed right tibiotarsal joint. Due to the severity of the sudden lameness, including the inability to extend the toes of the affected leg, radiographs were taken and the bird treated with antibiotics. Improvement was seen initially, but severe unilateral lameness persisted, which continued to affect the neurological exam for

the remainder of the study. Necropsy revealed that the tendon of the cranial tibial muscle and the long digital extensor tendon were swollen and discolored at the level of the tibiotarsal joint. The joint itself did not seem severely affected.

Bird # O-17 became ill during the first few weeks in captivity and developed mild respiratory signs and inappetence. This bird was isolated and treated with antibiotics and, after a slow recovery, seemed well enough to remain in the study. With time, however, it was apparent that O-17 was not going to be able to fly. She could flap her wings and spread them to dry but had a limited range of motion. This bird also lacked a good appetite, tended to stay isolated from the other birds, and on occasion was seen "shivering" or trembling while resting. In spite of some underlying abnormalities, this bird could swim well, jump, and run quite fast on the ground. On necropsy, both shoulder joints were inflamed and swollen with marked degenerative changes. There were erosions of the articular surfaces bilaterally, but the right shoulder was more severely affected. The right shoulder also had excessive pale yellow joint fluid and fibrin tags within the joint space. Additionally, cervical and cranial thoracic vertebral abnormalities were present. In spite of these problems, experimental data from this bird was still included in the analysis.

As a general maintenance procedure, birds had their feet checked several times weekly for any indication of problems. During the first few weeks, some birds developed abrasive lesions on their toe pads. Birds with the worst lesions tended to cling and climb on the cage wire. Abrasions were effectively treated with topical betadine solution, and the foot problems resolved within the first few weeks.

Discussion

Since the cormorants collected ranged between 2 and 4 weeks of age, staggering of dosing and testing was performed over a 3-week period with the oldest birds first, so that testing began at roughly the same age, 8-9 weeks of age. The close matching of ages was an important factor in evaluating growth curves and consumption rates. The growth curves and consumption rates were roughly comparable to those reported for other captive double-crested cormorants (DCCO)(DeLaRonde and Greichus, 1972). DeLaRonde and Greichus (1972), however, reported a growth rate which peaked around 6-7 weeks of age at about 1500 g for females and about 1700 g for males, then declined for the next five weeks to 1200 g for females and 1400 g for males. No specific reason was given, in that report, for the decline in body weights that was described; however, infection with aspergillosis could have been a factor. In the present study there was a flattening of the growth curve at 6-7 weeks (corresponding to 2-3 weeks before dosing), then a more slowly increasing curve after that (Figure 4.2). There is some variability in body weight depending on the subspecies of DCCO which may account for these differences. In general, body weights in the present study were in agreement with those reported for free-ranging specimens, as summarized by Johnsgard (1993), particularly when compared to reported values for *Phalacrocorax auritus floridanus*, one of the smaller subspecies found locally year-round in Florida.

Johnsgard (1993) estimated consumption rates for an adult DCCO at about 240 g per day or approximately 10% of average body weight. The higher rate of 15-20% of

body weight, in the present study, may reflect the increased needs of young growing birds compared to adults. The readily available food in captivity may also have led to slightly higher intake levels compared to that of free-ranging birds which forage for their food.

In the present study there was essentially no effect of treatment on either growth or food consumption. This finding contrasts with a number of reports which found decreased food intake and/or decreased body weights with exposure of birds to methylmercury (Frederick et al., 1997; Hill and Soares, 1987 Bhatnagar et al., 1982; Finley et al., 1979; Fimreite and Karstad, 1971; Fimreite, 1971; Borg et al., 1970). Given the neurological deficits produced by methylmercury, it is not always clear if decreased food intake is a result of lack of appetite or due to an inability to grasp and/or manipulate food. Fimreite and Karstad (1971) reported that hawks, which developed neurological signs when they were dosed with 7.2 or 10 ppm MeHg, retained their appetite but had difficulty eating.

In general, few studies of methylmercury in birds report no weight or food differences. Dieter and Ludke (1975) reported no effect on body weight changes or on final body weight, but did not discuss consumption rates. Kling and Soares (1978) found no effect on weight gain or feed consumption when quail were treated with methylmercury chloride at 5 or 15 ppm in the diet, although in the same study, birds treated with inorganic mercury as $HgCl_2$ (285 ppm) ate less and gained less weight than controls. Heinz (1979) reported no effect of 0.5 ppm dietary MeHg on weights or weight changes, but indicated a higher food consumption for the mercury-treated group in the second generation of this study. No difference was seen in the third generation, and the author

recommended viewing this result with caution. The variable outcomes with regard to these parameters may be related to species differences, as well as to dosing protocols. In the majority of avian studies, methylmercury has been administered orally in the diet, but the specific compounds used, the dose, dosing interval, and duration of dosing have been varied from one study to the next.

Although decreased food consumption, lack of appetite, and loss of weight are frequently cited findings for mercury toxicity in birds, the importance of this finding with regard to low level exposure, or as an early indicator of methylmercury exposure is unclear. The majority of the studies, which described feeding and weight as having been affected, used very high levels of exposure (many well in excess of 10 ppm dietary MeHg), and most often decreased feeding and wight were found at or after onset of considerable neurological impairment. Most reports agreed that the neurological signs in birds are progressive and irreversible when detected clinically. These birds usually succumbed within a short time after having developed overt neurological manifestations.

The high-dose level of 3.5 mg MeHg/kg diet in this study was selected as a level that would be borderline clinical (i.e. a level approaching clinical toxicity with the potential appearance of very early clinical signs). This condition was achieved and the cormorants developed only very subtle neurologic deficits in the last few weeks of the study (Chapter 8). Considering the lack of overt neurological signs in the cormorants, it was not surprising that lowered weight or consumption levels were not found.

The slight decrease in body weights noted in females of group C (low dose) was significant when compared to group B but not controls. This finding should be interpreted

with caution, since it was likely the result of a confounding variable. Due to the staggering of treatments, and subsequent euthanasia, the number of birds progressively diminished during the last three weeks of the study. The decrease in population size seemed to disturb the social structure, especially at the end when only 5 birds remained. The remaining birds began to act strangely, becoming anxious, flighty, and uninterested in food. Three birds remained by themselves over the last weekend and thus had the longest time span, during the eighth week of treatment, under the "altered" social structure. These three, two of which were from group C, also exhibited the greatest decline in weight and food consumption. This finding may be noteworthy, especially for future projects with captive cormorants, where the highly social nature of these birds may potentially affect the outcome of the study.

CHAPTER 5
BLOOD, TISSUE, AND FECAL-URATE MERCURY DISTRIBUTION

Introduction

Methylmercury readily enters and accumulates in most tissues. The purpose of this section is to quantify the levels of total mercury in internal organs, blood, and fecal-urate matter of Double-crested cormorants dosed with methylmercury, and to evaluate relationships between Hg levels in these media. The three treatment groups consisted of group A (control), B (high), and C (low). Groups B and C were given 3.5 and 0.5 mg/kg daily dietary methylmercury chloride, respectively, as described in detail in Chapter 3.

Methods

Sample Preparation

Samples of tissues, blood, and feces were collected from 22 cormorants, each dosed for eight weeks with one of three treatments: Control, a low dose (0.5 mg/kg) or a high dose (3.5 mg/kg) of dietary methylmercury. Blood was collected from the ulnar vein of each animal into lithium heparin tubes, at the end of each week of treatment. Due to the clotting tendency of avian blood and the difficulty of removing a representative sample from the narrow blood tubes, at least 1 ml of whole blood was measured and transferred to 20 ml glass vials with teflon coated screw tops. If homogenation of the blood sample

proved difficult then the sample would be predigested with acid in its storage vessel. Blood was stored frozen until analysis. Although clotting of the blood samples occurred, they were sufficiently homogenized so that predigestion was unnecessary.

Tissue samples from liver, kidney, spleen, muscle, eye, and brain were collected at postmortem examination and stored frozen in Whirl-pack bags. One whole eye, with all extraocular structures removed, was collected from each subject. Analysis of specific ocular components such as the retina, lens, or vitreous was not practical. The entire left cerebral hemisphere and optic lobe were saved from each bird. Initial attempts to include half of the cerebellum led to compromise of the segments saved for histology, hence the cerebellum from only one bird was included.

Individual fecal-urate samples were collected at the end of each treatment week by placing each bird in an isolated cage with a clean plastic liner. All samples were collected between 8 and 10 am. Several afternoon samples were also collected from each treatment at the end of the last week to evaluate diurnal differences. At least 1 g of fecal-urate material per sample was stored frozen in whirl-pack bags for later analysis. Three duplicate samples from each group, at the end of weeks 4 and 8, were oven-dried, desiccated, and analyzed as dry weight.

Prior to digestion tissues were thawed, and 3-5g samples were homogenized by fine mincing and mixing using disposable razor blades. Blood samples were homogenized by sonication for 5 minutes followed by vortexing. The liquid nature of the fecal-urate material required only thorough mixing for adequate homogenation.

Sample Digestion and Analysis

Sample digestion and mercury analysis were performed according to modified FDEP protocols (MT-014-1 and MT-010-1). Analyses were grouped by tissue type or, for blood and feces, by treatment week. All reagents and solvents were graded suitable for mercury analysis. An amount of 0.200 to 0.300 gram of each sample was weighed and placed in 125 ml Nalgene® wide-mouth polyethylene disposable bottles. Between 0.1000 and 0.1500g of DOLT-2 reference material was weighed in 250 ml wide mouth glass bottles (I-CHEM Superfund-Analyzed™ , Fisher). Glass was used for the DOLT-2, which has a powdery consistency, to reduce variability in weighing which resulted from static in the plastic containers.

Samples were digested with 5ml concentrated sulfuric acid (H_2SO_4) and 2 ml of concentrated nitric acid (HNO_3) for 5 minutes. Samples were heated in a 56 - 58 °C water bath for 30 minutes then allowed to cool to room temperature. Twenty ml of Optima grade deionized (DI) water was added to tissue samples followed by 10 ml of 8% potassium permanganate ($KMnO_4$, J.T.Baker, Philipsburg, NJ). Solutions were allowed to react for 10-15 minutes. If color faded, more $KMnO_4$ was added in 1 ml aliquots until a dark purple/brown color persisted. Four ml of fresh 5% potassium persulfate ($K_2S_2O_8$, Fisher) were added, and the digestion containers were capped and gently inverted several times and then the lids were loosened slightly to release pressure. Following overnight digestion, preparation for analysis was completed by adding 4 ml of 12% hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) and 1 ml of 3% antifoaming A emulsion (Sigma, St. Louis, Mo). Samples were all brought up to 100 ml total volume with DI water, sonicated for

10-15 minutes, then transferred to borosilicated glass culture tubes to be analyzed. Solutions containing any particulate matter were filtered with 0.45μm acrodisk filters.

Total mercury levels were determined by cold vapor atomic absorption spectrophotometry using the commercial Thermo Separation Products Mercury Analyzer (Riviera Beach, FL). The Hg^{2+} in the samples was reduced to Hg^0 by 2% stannous chloride in 10% sulfuric acid. Elemental mercury was bubbled from solution and then was analyzed by atomic absorption at 253.7 nm (low pressure mercury source lamp).

Quality Control and Statistics

Calibration curves, prior to each set of samples, consisted of a 2% nitric acid and 1% sulfuric blank and four or five standard solutions including 0.2, 0.5, 1, 5, and 10 ppb of mercuric chloride in 2% HNO_3 and 1% H_2SO_4. Each set of samples processed also included at least one sample duplicate. Raw results were adjusted by digestion blank subtraction and were compared against two spiked solutions and/or a dogfish liver certified reference material (DOLT-2, National Research Council, Ottawa, Canada). The spikes consisted of 5 ppb $HgCl_2$ and MeHgCl solutions which were made with 5 μl of 100 ppm $HgCl_2$ or MeHgCl in 20 ml of DI water, followed by routine digestion along with the other samples.

The R^2 for calibration curves ranged from 0.9882 to 0.9999. The practical detection limit was 0.02 ppb and duplicates were within 2.5 %RSD on average (range 0.62 - 6.57%). DOLT-2 and spike recovery ranges were 87 - 113 % and 94 - 112 % respectively. All results were reported as wet weight.

Background levels in blood collection tubes and in glass and plastic storage containers were tested by freezing 1 ml of DI water in each container type and then analyzing for mercury. Background levels for the diet included measurement of mercury in whole, scaled sardines and whole silverside. Capelin, shrimp, and squid samples were lost, but an analysis of capelin from the same shipment was conducted at another lab and found to be 0.046 mg/kg (Frederick, et al. 1997).

Results were reported as wet weight except as noted for selected fecal-urate samples. All mercury data was log transformed. Tissue data was analyzed using one way ANOVA and blood and feces were analyzed using repeated measures ANOVA. Tukey-Kramer multiple pairwise comparisons were performed on all sets and the significance levels set at $p \leq 0.05$. A paired t-test was used to compare morning and afternoon fecal-urate samples. Correlations between blood, tissues and feces were assessed using Quatro Pro 8.0; all other tests used SAS 6.1.

Results

Mercury levels in the fish diet (±1SD) were 0.0237 (± 0.0081) mg/kg and 0.0305 (± 0.0135) mg/kg in sardines and silversides respectively. Levels in storage media were below the detection limit (≤0.02 ppb). Tissue, blood, and fecal total mercury levels have been summarized in Tables 5.1 and 5.2.

Blood mercury accumulated linearly on both dose groups, although the linear fit for the low-dose group was not as good as for the high-dose (Figure 5.1). For groups B and C each successive 2 week measurement was significantly different from the previous

measurement. Control bird Hg levels in the blood were very low compared to dosed birds (Figure 5.1), but within this group, levels at weeks 4 and 8 were significantly higher than at the baseline (≤ 0.05). There was no difference between any of the groups at the baseline time. Blood Hg level was linearly correlated to the cumulative dose of MeHg administered with an R^2 of 0.9661 (Figure 5.2).

Liver, followed by kidney, had the highest total mercury concentrations in both high- and low-dose groups (Figure 5.3). Average liver Hg concentrations were 58.5 (± 5.5) and 9.8 (± 1.7) μg/g for groups B and C, respectively. Averages for kidney, spleen, and muscle were all within 20 - 30 μg/g in group B, and 1 - 4 μg/g in group C. The eyes also had a significant increase in Hg compared to controls, but concentrations were quite low compared to other tissues.

Brain levels for combined cerebrum and optic lobe measurements averaged 11.1 (± 1.7) μg/g and 2.4 (± 0.3) μg/g for group B and C, respectively (Figure 5.4). The variations between different brain regions were not significant. The one cerebellum that was analyzed had slightly lower Hg content than the other brain parts. All tissue Hg levels, including brain, were linearly correlated to blood levels at the end of treatment (Figure 5.5 and 5.6). Tissue levels were similarly correlated to the total dose of MeHg administered.

Fecal-urate Hg levels (Figure 5.7) also increased linearly over time, especially in the high-dose group. Both mercury treated groups had fecal-urate Hg levels that were significantly higher than controls at all times except baseline. Levels in group C increased slightly between times 0 and 2 weeks, but remained at about 0.06 - 0.08 μg/g for the remainder of the study. Fecal-urate excretion reached 1.1± 0.8 μg/g after 8 weeks at the 3.5 mg/kg dietary MeHg dose. Although there was a linear trend, fecal-urate Hg did not

correlate well with blood ($R^2 = 0.6177$) or the dose administered ($R^2 = 0.6082$). When evaluated for blood levels of < 25 µg/g the linear relationship with fecal Hg was much stronger with an $R^2 = 0.8551$. Above these blood levels, fecal-urate concentrations became quite variable, with a distinct separation between those birds excreting higher levels and those excreting less (Figure 5.8).

Dry weight fecal-urate samples for three birds in each group, at weeks 4 and 8, are presented in Table 5.3. Morning and afternoon sample comparisons are shown in Table 5.4. Average fecal moisture content was 91.2 ± 4.7 % (range: 82-97 %) for all dry weight samples. Mercury levels were a factor of 4 - 7.5 times higher when evaluated as dry weight than as wet weight.

Discussion

The high dose level of 3.5 mg MeHg/kg diet daily for 8 weeks resulted in high blood and tissue levels while the 0.5 mg/kg dose resulted in much lower accumulation of mercury. Liver levels of 30 - 40 µg/g, and brain levels of 10 - 15 µg/g have been associated with overt neurotoxicity (Scheuhammer, 1988). The birds in the high dose group in the current study had 48 - 66 and 8.5 - 14.5 µg/g Hg in liver and brain respectively, and did not exhibit the typical signs of weakness, staggering, and incoordination. During the neurologic exams (Chapter 8), proprioceptive deficits were detected in the high dose group starting at 5 weeks of dosing, but these were not readily apparent by casual observation of the birds. Visual evoked potentials were also affected after 4 weeks of dosing (Chapter 7).

Table 5.1 : Blood and fecal-urate total Hg concentrations in µg/g (ppm), at two-week intervals. A - control, B - 3.5, and C - 0.5 mg MeHgCl/kg diet/day.

	Week	Tx	n	Avg.	Std.	%RSD	Low	High
Blood	Wk0	A	7	0.165	0.031	19.0	0.124	0.219
		B	8	0.163	0.030	18.4	0.136	0.216
		C	7	0.130	0.032	24.6	0.081	0.181
	Wk2	A	7	0.185	0.032	17.1	0.144	0.243
		B	8	8.210	1.639	20.0	6.447	11.12
		C	7	1.028	0.103	10.0	0.853	1.187
	Wk4	A	7	0.356	0.092	25.8	0.156	0.426
		B	8	19.035	1.935	10.2	16.515	21.980
		C	7	3.256	0.455	14.0	2.216	3.793
	Wk6	A	7	0.214	0.021	9.7	0.185	0.246
		B	8	26.569	3.025	11.4	22.154	32.074
		C	7	2.203	0.247	11.2	1.797	2.606
	Wk8	A	7	0.292	0.045	15.4	0.239	0.370
		B	8	39.737	5.528	13.9	31.855	48.267
		C	7	4.769	1.334	28.0	3.580	7.667
Feces	Wk 0	A	6	0.015	0.012	81.3	0.002	0.035
		B	8	0.010	0.008	76.4	0.003	0.027
		C	7	0.010	0.007	75.2	0.004	0.025
	Wk 2	A	7	0.011	0.009	82.9	0.003	0.030
		B	8	0.255	0.093	36.3	0.150	0.439
		C	7	0.076	0.073	96.5	0.015	0.240
	Wk 4	A	6	0.014	0.012	85.3	0.005	0.038
		B	6	0.489	0.109	22.3	0.374	0.711
		C	6	0.067	0.035	52.1	0.024	0.131
	Wk 6	A	7	0.016	0.011	69.8	0.004	0.041
		B	8	0.679	0.295	43.4	0.294	1.149
		C	7	0.061	0.023	37.5	0.023	0.091
	Wk 8	A	7	0.017	0.006	36.1	0.009	0.031
		B	8	1.112	0.845	76.0	0.465	2.493
		C	7	0.081	0.017	20.8	0.060	0.107

Table 5.2 : Tissue total Hg concentrations in µg/g (ppm) for three treatment groups, at the end of 8 weeks of dosing with methylmercury. Treatments (Tx) A, B, and C are controls, high (3.5 mg/kg), and low (0.5 mg/kg) dose, respectively.

Tissue	Tx	n	Avg.	Std.	%RSD	Low	High
Liver	A	7	0.849	0.186	21.9	0.678	1.269
	B	8	58.518	5.524	9.4	48.200	66.352
	C	7	9.782	1.688	17.3	7.464	12.996
Kidney	A	7	0.271	0.079	29.2	0.211	0.457
	B	8	28.741	4.145	14.4	20.466	35.295
	C	7	3.699	0.520	14.1	2.948	4.587
Muscle	A	7	0.154	0.024	15.4	0.126	0.194
	B	8	21.492	3.065	14.3	17.348	25.652
	C	7	2.210	0.554	25.1	1.632	3.394
Spleen	A	7	0.213	0.099	46.3	0.146	0.452
	B	8	25.815	3.414	13.2	20.650	30.908
	C	7	1.462	0.363	24.8	0.947	2.065
Eye	A	7	0.025	0.006	24.7	0.019	0.04
	B	8	2.538	0.700	27.6	1.842	3.942
	C	7	0.374	0.036	9.6	0.331	0.445
Cerebrum + Optic lobe	A	4	0.086	0.009	10.3	0.071	0.093
	B	5	11.084	1.654	14.9	8.504	13.676
	C	4	2.393	0.324	13.6	2.035	2.885
Cerebrum	A	3	0.101	0.022	22.3	0.081	0.132
	B	3	13.326	1.259	9.5	11.579	14.500
	C	3	2.807	0.568	20.2	2.063	3.442
Optic Lobe	A	3	0.081	0.006	7.7	0.072	0.086
	B	3	11.910	2.127	17.9	8.902	13.462
	C	3	2.227	0.476	21.4	1.605	2.761
Cerebellum	A	—	—	—	—	—	—
	B	1	9.577	0	0.0	9.577	9.577
	C	—	—	—	—	—	—

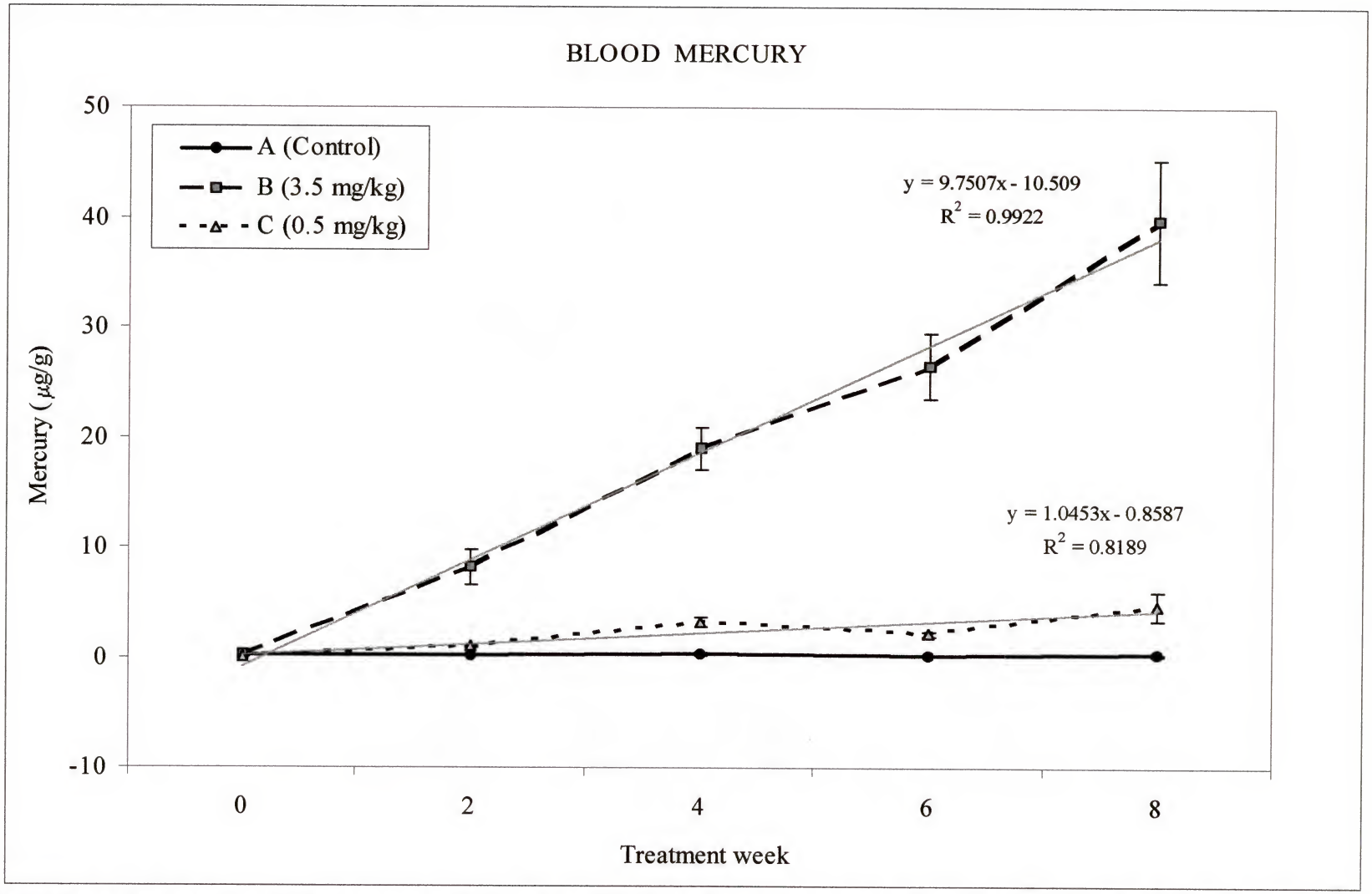


Figure 5.1 : Total blood mercury in μg/g (ppm), for 3 treatment groups, at 2-week intervals (Avg. ± 1SD). Note that R^2 for low dose is 0.8189 for a linear relationship. Alternatively, a better fit may be obtained by $y = 0.1733x^{2.1479}$ with an $R^2 = 0.9135$.

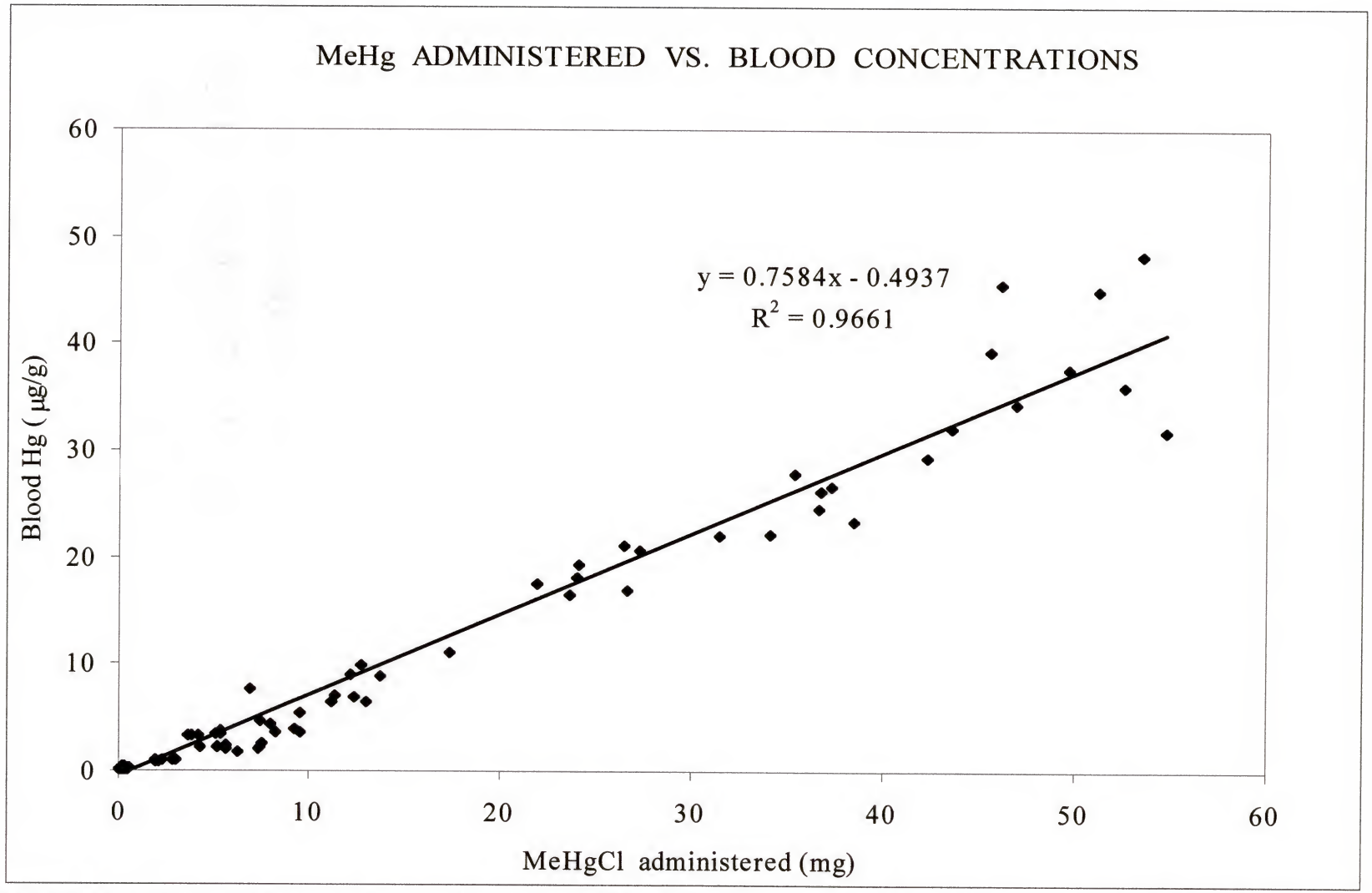


Figure 5.2 : Correlation between the cumulative dose of MeHgCl administered and blood Hg concentrations. Plot includes data from treatments A, B, and C.

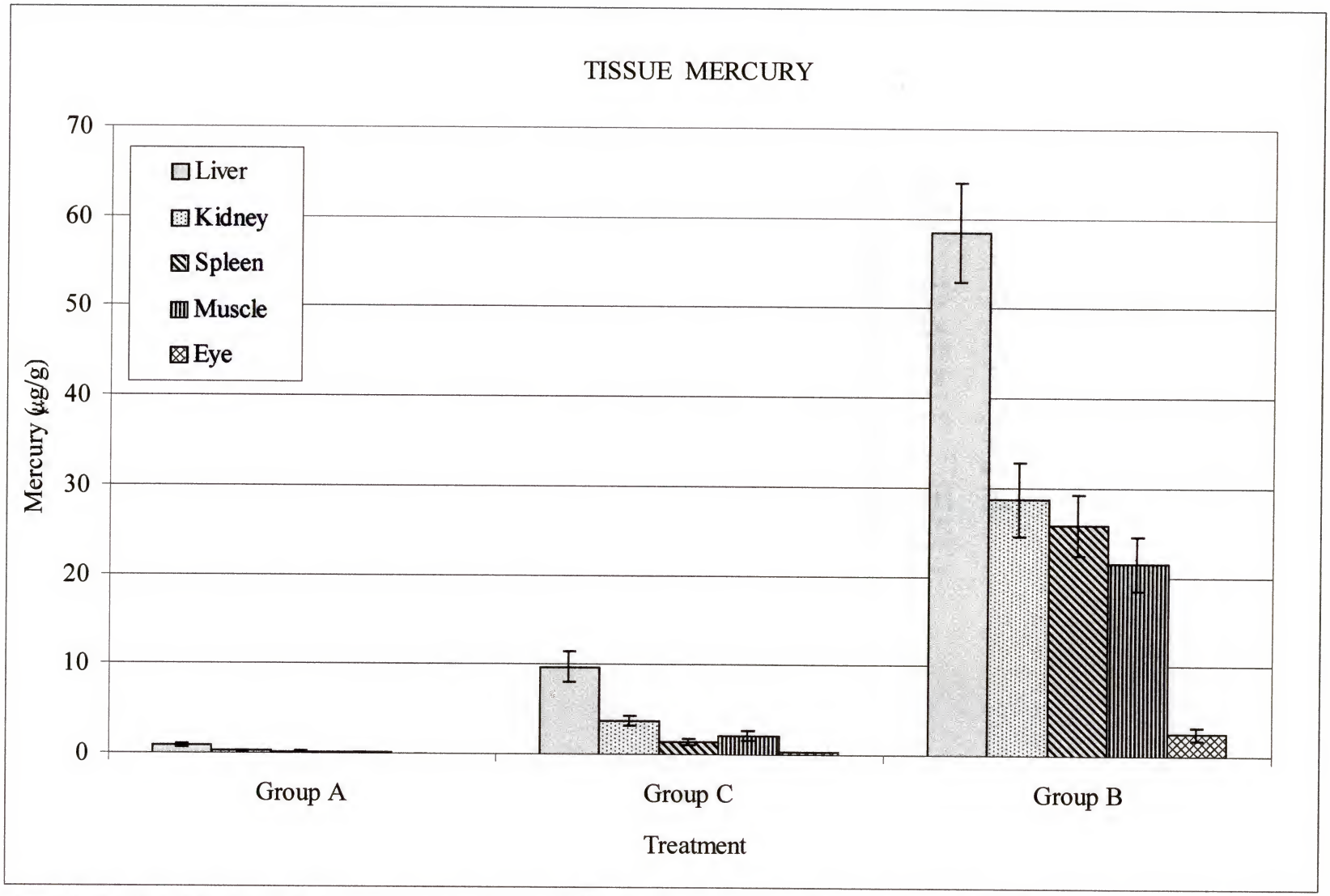


Figure 5.3 : Total Hg levels in tissues (µg/g as wet weight) for three treatment groups (Avg. ± 1SD).

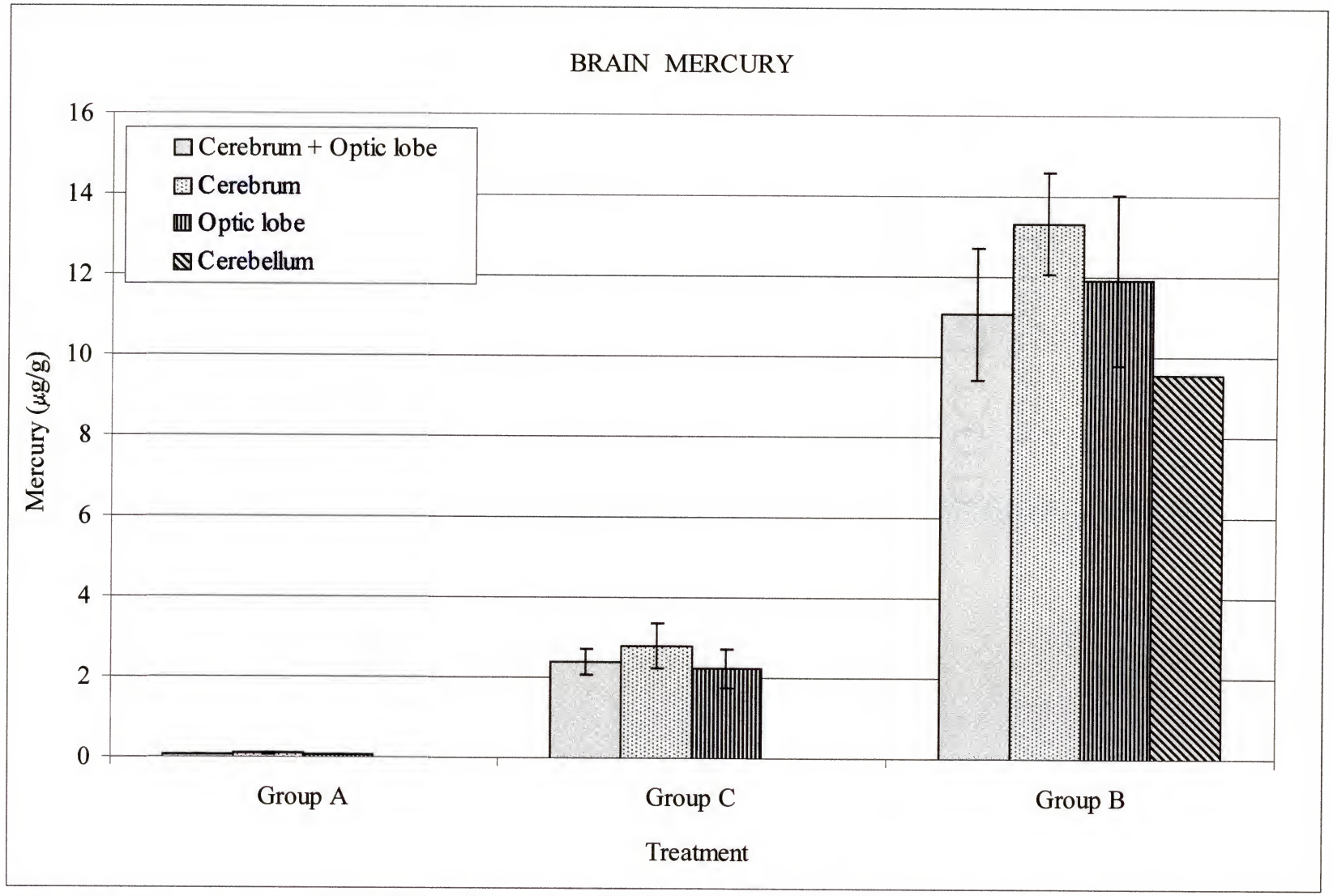


Figure 5.4 : Brain Hg content (µg/g as wet weight), for various brain regions in three treatment groups (Avg. ± 1SD).

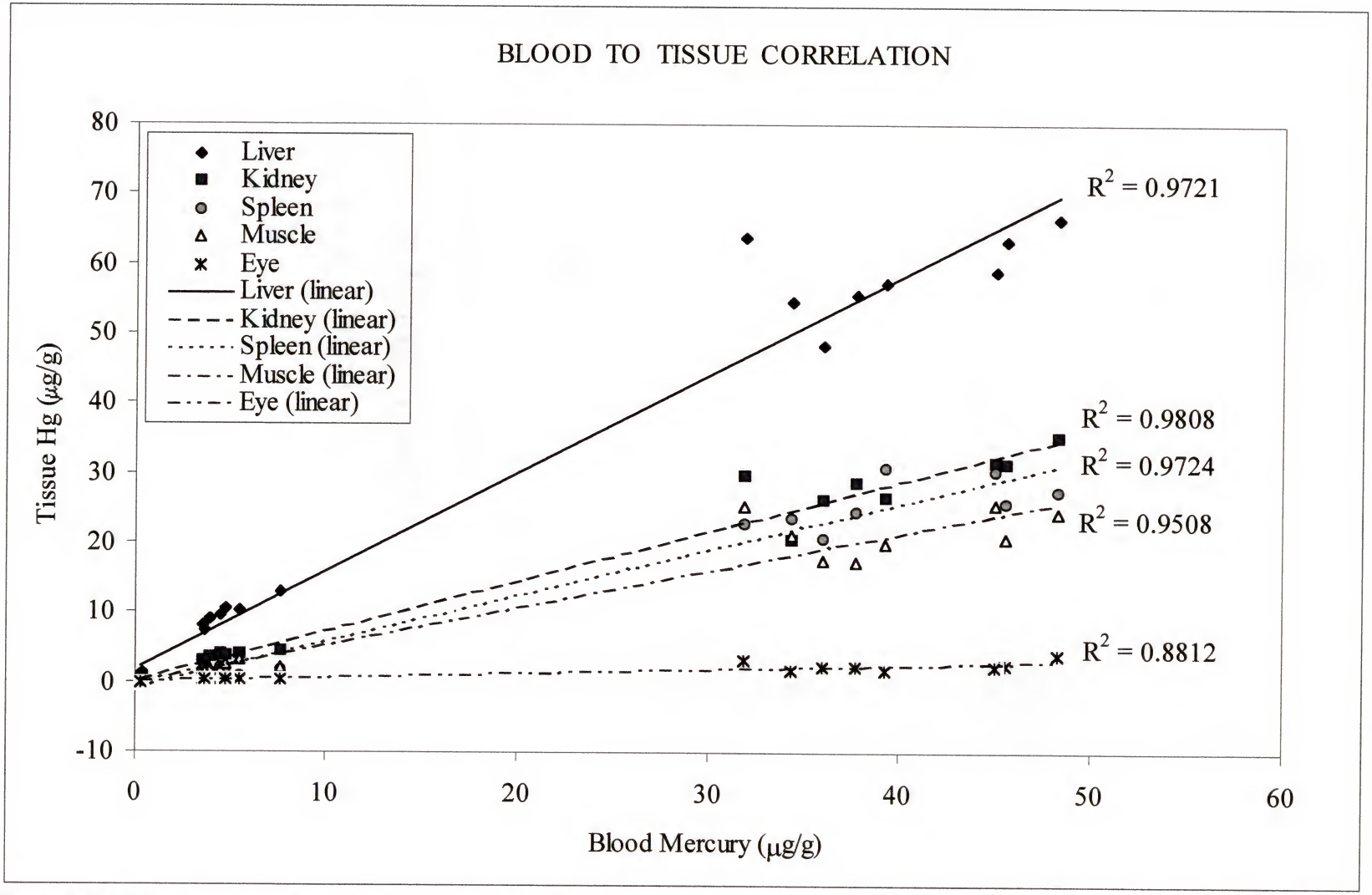


Figure 5.5 : Linear correlations between blood Hg and tissue Hg levels at the end of dosing. The plots include data from all three groups – control, low, and high doses.

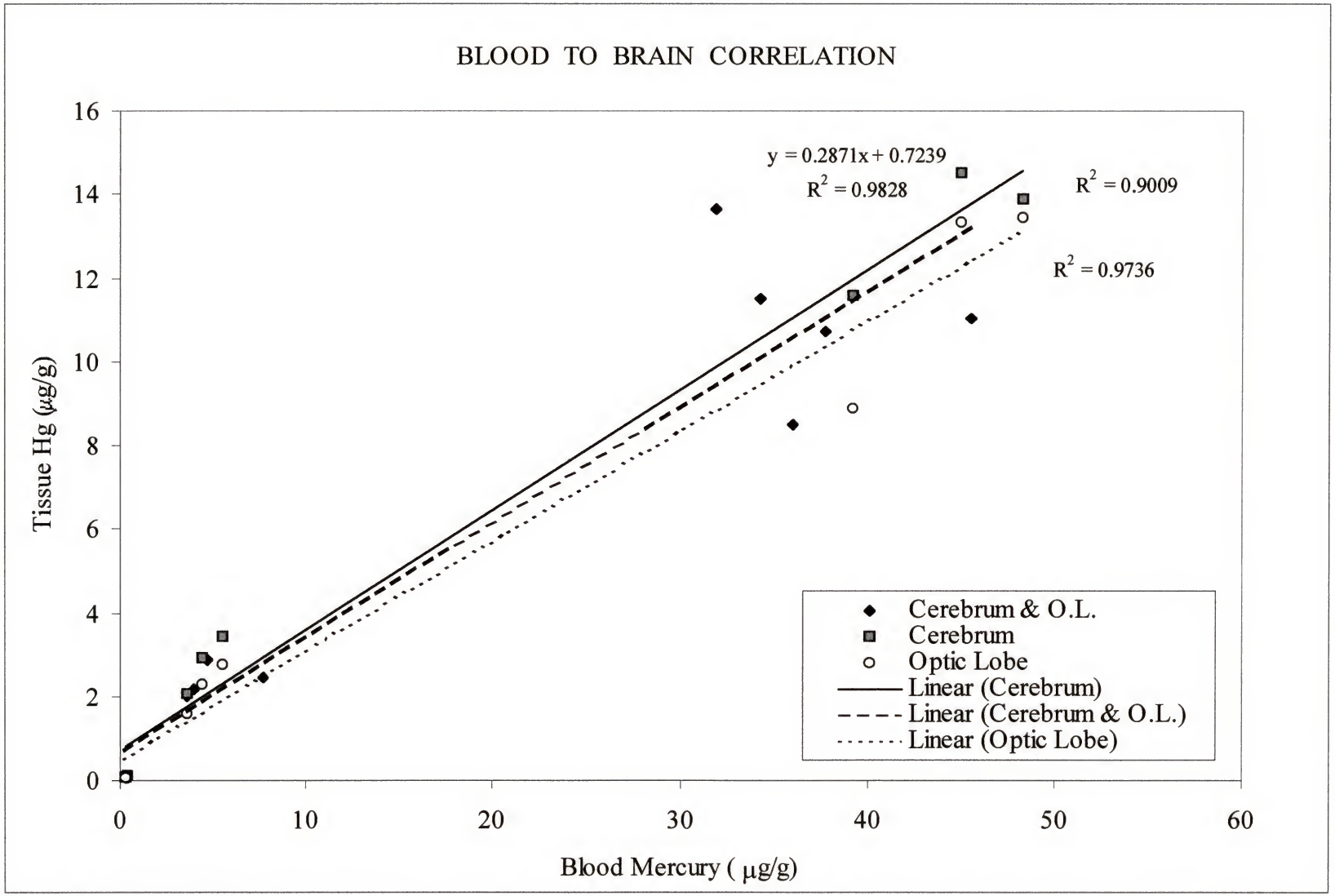


Figure 5.6 : Brain to blood mercury correlations. Relationships include data from all treatment groups (control, low, and high dose).

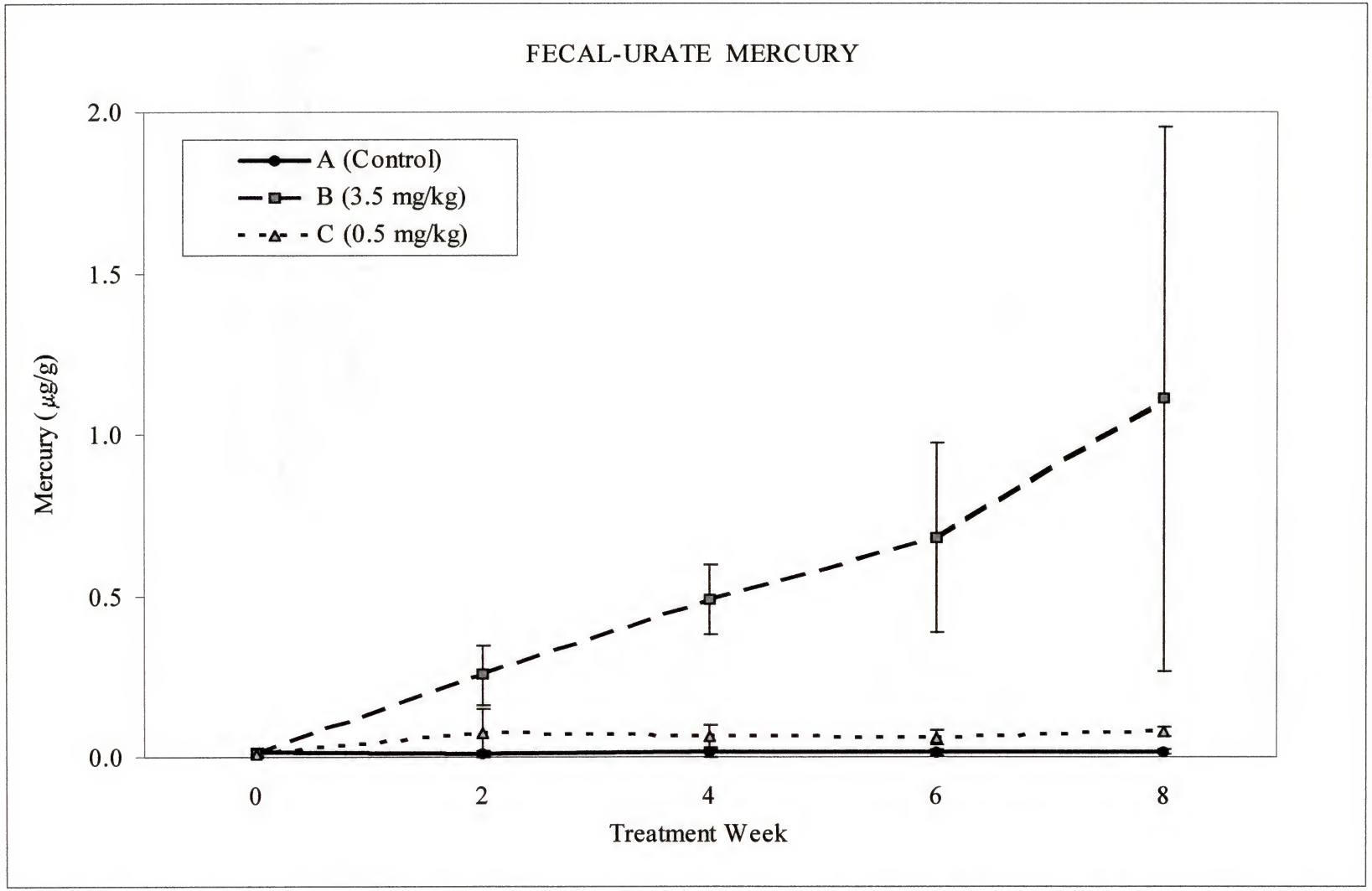


Figure 5.7 : Fecal-urate Hg concentrations ±1SD (µg/g) at the end of 2-week intervals. At weeks 2, 4, 6, and 8 averages for all groups were significantly different from each other at those time points ($p<0.05$).

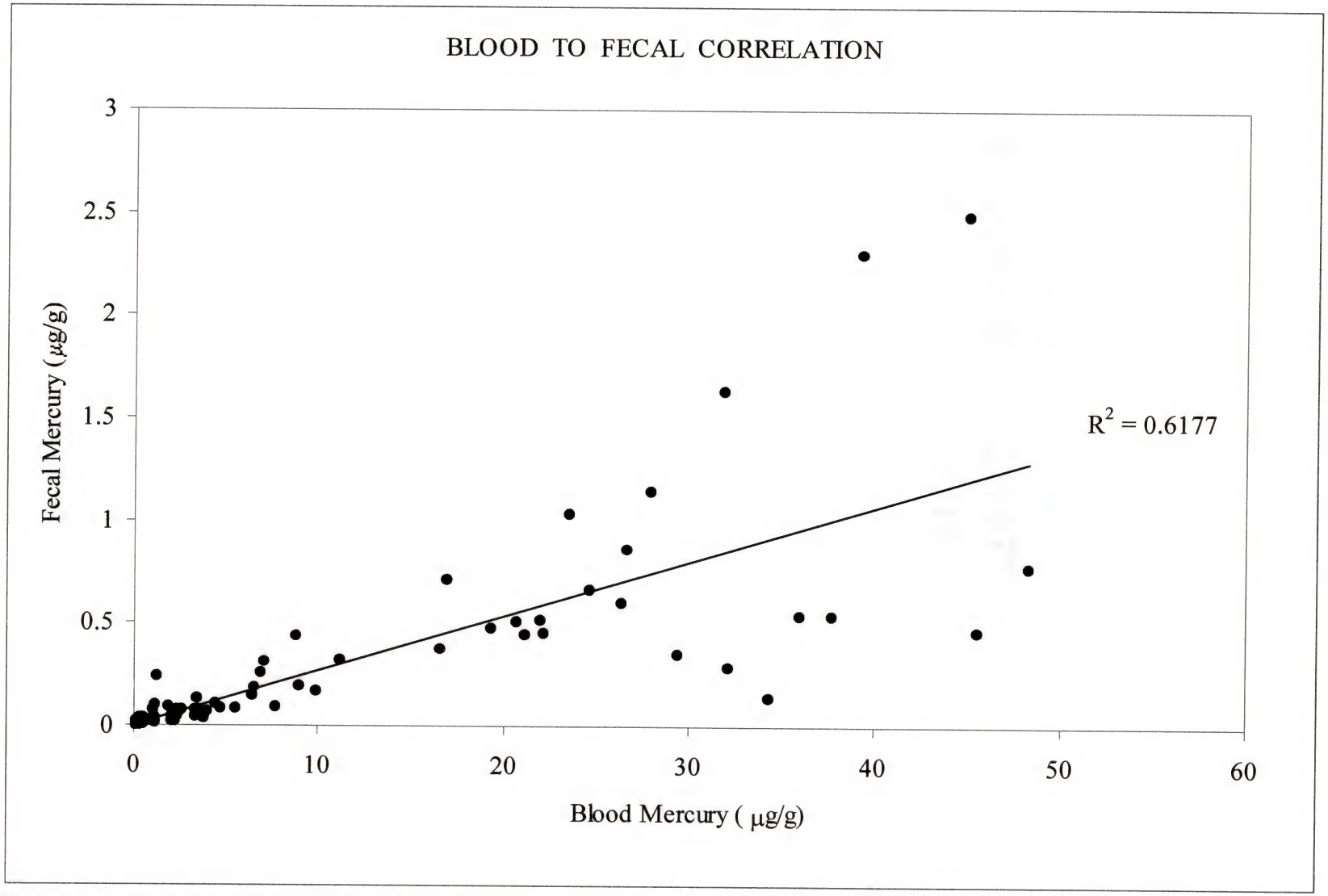


Figure 5.8 : Correlation between blood and fecal Hg content. Plot includes data from all three treatments.

Table 5.3 : Fecal-urate Hg concentrations as dry weight (μg/g ± 1 SD) in selected samples. A = control, B = 3.5, and C = 0.5 mg MeHg/kg diet.

Week	Tx	n	Avg.	Std.	Low	High
Week 4	A	3	0.158	0.151	0.018	0.367
	B	3	2.304	0.502	1.600	2.684
	C	3	0.282	0.192	0.136	0.554
Week 8	A	3	0.063	0.015	0.048	0.083
	B	3	3.353	2.110	1.026	6.138
	C	3	0.607	0.468	0.242	1.268

Table 5.4 : Fecal-urate Hg concentrations in paired morning and afternoon samples (μg/g ± 1 SD, as wet weight). A = control, B = 3.5, and C = 0.5 mg MeHg/kg diet.

	Tx	Time	n	Avg.	Std.	Low	High
Week 8	A	AM	3	0.020	0.008	0.015	0.031
	A	PM	3	0.014	0.006	0.006	0.021
	B	AM	3	0.408	0.190	0.140	0.544
	B	PM	3	0.192	0.070	0.124	0.288
	C	AM	3	0.063	0.004	0.060	0.068
	C	PM	3	0.089	0.045	0.029	0.138

Low concentrations of tissue Hg have also been associated with adverse effects, especially developmental and behavioral. Fimreite (1971) reported reduced egg production and reduced hatchability in MeHg treated pheasants which accumulated ≤13.7 ppm mercury in their livers (mean values 0.59 - 7.83 ppm). In three generations of mallard ducks fed 0.5 ppm of MeHg in their feed, brain Hg levels remained below 1 ppm, and liver and kidney levels below 2 ppm Heinz (1979). Female ducks in that study laid greater numbers of eggs outside their nestboxes, laid fewer eggs and produced fewer ducklings. Additionally, ducklings from parents fed 0.5 ppm MeHg had altered behavioral responses (Heinz, 1979). Barr (1986) found that reduced egg laying and abnormal nesting behavior were associated with mean Hg concentrations of 0.3 - 0.4 ppm in prey items and 2 -3 ppm in the brains and eggs of adult loons. In the current study even the low dose group accumulated tissue levels within the ranges mentioned above.

One explanation for the difference in sensitivity to MeHg may relate to selenium levels. Selenium is known to provide a protective effect against MeHg toxicity (Stoewsand et al., 1974), and the diet of marine species of birds may provide them with relatively higher selenium levels. The selenium status was not included in this study, but it may ultimately be useful to determined these levels for comparison to other studies and for further elucidation of mechanisms and interactions of MeHg.

Differences in species, age, and dosing protocols among the various studies may also account for the perceived variability in sensitivity among birds. Alternatively, it is possible that toxicity may begin at about the same blood or tissue levels in different species, but it may present as subtle or gradual alterations that are not easily detectable.

By extrapolating between week 4 and 6 for week 5, the blood level at which the proprioceptive deficits were detected in cormorant can be estimated to be about 22-23 µg/g. Since tissue Hg concentrations were directly proportional to blood levels, further extrapolation (using Figure5.5), would lead to an estimated liver concentration of about 30 - 35 µg/g at the time when the proprioceptive deficits first became apparent. Likewise, VEP amplitudes were decreased as early as 4 weeks, which means that brain Hg concentrations at which toxicity begins is considerably less than what was measured after 8 weeks of dosing (Chapter 7). Ideally, one would like to conduct a dosing study which includes tissue Hg measurements at multiple time points in order to accurately ascertain the tissue concentrations at which a functional deficit is first detected.

At the dose and duration that MeHg was administered in this study, tissues accumulated Hg in a linear fashion relative to the total dose administered. Scheuhammer (1988) also found a similar linear relationship of Hg accumulation in tissues of zebra finch. This relationship, however, is a function of the dose and duration of the study and indicates that a steady state blood level was not reached. In contrast to the high dose group, the rate of increase in blood Hg concentration in the low dose cormorants slowed at around 4 weeks. To have reached a steady state blood level in the high dose birds would have required extending the project by at least several weeks. Since the birds dosed with 3.5 mg/kg MeHg in their diet were already showing early neurologic changes (Chapters 7 and 8), it is unlikely that they would have survived for an extended period.

Within the control group a small, but significant, increase was seen in blood Hg level. The slight increase was statistically significant at weeks 4 and 8 compared to

baseline time 0. This finding may have reflected exposure to small amounts of mercury from the diet, since the sardines and other fish had a small amount of background mercury in them.

While fecal-urate Hg levels were quite low relative to other tissues, it was hoped that perhaps assessment of individual fecal samples would provide an added means to non-invasively monitor exposure in wild birds. The purpose here was not to determine total fecal excretion rates, but to determine if there is a relationship between individual fecal Hg levels and blood or tissue levels. In spite of the low levels of Hg found in the feces, especially in the low dose group, the average Hg content in both treated groups was significantly different from controls and from each other. Thus, a fecal-urate value of 0.06 ppm, the lowest average fecal Hg level for group C during treatment, was indicative of exposure to mercury above background levels. Unfortunately a low fecal Hg content cannot rule out a low-level mercury exposure since in group C there were several birds with fecal Hg levels within the control ranges during all weeks except week 8. However, at the 3.5 mg MeHg/kg fish/day dose level, by week 2 the lowest measured fecal Hg concentration was 0.15 μg/g which is 5 times the highest control level measured.

Thus, wet fecal Hg levels above 0.15 ppm (or even as low as 0.06 ppm) may be associated with intake of Hg at low levels or short-term intake at higher levels (ex. 2 weeks @ 3.5 mg/kg). A generalization could be made that levels ≥ 0.25 μg/g in wet fecal-urate material is likely to be related to intake of mercruy at relatively high doses, even for a short time. Additionally, for blood levels of < 25 μg/g a fairly good linear relationship existed between blood and fecal Hg; above this level the linearity of the relationship did not hold. Caution should be used in applying these findings to field data because the

intermittent exposure and variable doses that free ranging birds are exposed to are likely to yield differing results. Furthermore, variables such as age, breeding status, and molting patterns, which were not incorporated into the present analysis, may also affect fecal-urate excretion (Honda et al., 1986; Furness et al., 1986).

Measuring dry fecal Hg may be of use in field monitoring of Hg exposure in birds and knowledge of dry weight values may reduce concerns about obtaining fresh feces. The initial assessment of dry weight fecal-urate values in cormorants indicated that this approach did not significantly reduce the variability seen in wet weight samples. However, further data would be needed to more clearly verify dry weight fecal-urate Hg ranges. Only a few samples were evaluated and recovery rates from dried samples raised questions regarding the accuracy of Hg levels obtained from the dry samples.

If it is assumed that all mercury in the feces is bound to solid material and does not volatilize during drying, then dry weight analysis may be accurate. However, if this assumption were true, then based on the calculated moisture content of the samples only 39 ± 20 % of what we would have expected in the dry samples was recovered. An oversight in the current study was the use of an appropriate control in the drying process, with a known amount of inorganic mercury, which would have verified if any Hg was lost. Most of the mercury in excrement after the ingestion of MeHg is in the inorganic form (Norseth and Clarkson, 1970) which may be more susceptible to volatilization than MeHg.

Drawbacks of the fecal analysis included a mixed sample containing both feces and urates which are eliminated together from the cloaca, potential diurnal variability, and increasing variability as blood Hg levels increased. The urate portion reflects renal excretion, which in humans is generally considered to be of smaller magnitude than the

biliary fecal route (Norseth and Clarkson, 1970). No data was available in birds regarding the relative renal excretion of Hg compared to fecal excretion rates. Ultimately, these types of issues lead to complexity when trying to assess the significance of combined fecal-urate Hg levels in birds.

There are practical implications of the data obtained from cormorants in this study. First, cormorants exposed to as little as 0.5 ppm MeHg in their food can accumulate tissue levels that have been associated with reproductive, developmental, and behavioral changes. In Florida this is a significant issue since fish from many parts of the state have 0.5 ppm of mercury or more (Ware et al., 1990). Second, at exposure levels of 3.5 ppm in their food, high tissue Hg levels accumulate and early neurotoxicity may develop. While extended exposure to 3.5 ppm MeHg in the wild may not be as likely as the lower level exposure, tissue Hg levels within the ranges obtained in this cormorant study have been measured in wild aquatic birds (Sundlof et al., 1994; Sepúlveda et al., 1998). Third, when a wild bird or carcass is tested for Hg content, this data provides a reference point from which one can estimate the likelihood of mercury contributing to clinical illness.

The relatively short duration of the study and use of a single age group were design limitations of this study which should be kept in mind when comparing data to free ranging birds. To determine what happens to blood and tissue levels long term, or with variable and intermittent exposure, would require an extended study of several months or more. Further studies evaluating MeHg exposure at intermittent intervals, variables doses, prolonged durations, different age groups, and during varied molting patterns as is more likely to occur in the wild, may provide additional data for comparisons between field and laboratory situations.

CHAPTER 6
PATHOLOGY

Introduction

Pathological effects of MeHg in birds have been described for a number of species (Pass et al., 1975; Borg et al., 1970; Heinz and Locke, 1976; Fimreite and Karstad, 1971), yet few systematic studies have evaluated this aspect in aquatic, fish-eating avian species. Data of pathological effects from a controlled study, on a species potentially at risk due to its dietary preferences, is an important component in compiling information on free-ranging species. Evaluation of the dose-response relationship with regards to pathology may provide valuable data which can contribute to knowledgeable assessment of environmental risk of methylmercury to wild birds.

The purpose of this section is to describe gross and histopathological changes, and to characterize mercury distribution within tissues, resulting from exposure of double-crested cormorants subchronically to methylmercury. Since these birds were collected from the wild, possible prior exposure to a variety of environmental factors presented a potential confounding element in evaluation of pathological changes. Hence, results relating to MeHg exposure are presented strictly in reference to control birds as the baseline reference point.

Methods

Necropsy and Tissue Collection

Birds were humanely killed with an intravenous sodium pentobarbital solution while they were under general anesthesia, at the end of the final electrophysiology testing. Complete necropsies were performed immediately following euthanasia. Gross pathological changes were recorded on individual necropsy forms then summarized by treatment group. Due to the sensitivity of brain and ocular tissues to autolysis, the head was examined first and these organs removed and stored promptly. Organs were measured and/or weighed intact. Organ weights as a percent of body mass, adjusted for gender, were arc sine transformed and analyzed with one way ANOVA and Tukey -Kramer multiple pairwise comparisons.

Samples from major organs were collected and stored in 10% neutral buffered formalin (10% NBF) for light microscopy, and frozen in whirl-pack bags for mercury analysis (Chapter 5). One globe was stored for total mercury analysis, while the other eye was hemisected and one section placed in 10% NBF for routine histology and the other in 2% glutaraldehyde for electron microscopy. Other tissues which were evaluated at the light microscopic level included one hemisphere of the brain, cervical spinal cord, sciatic nerve and/or the brachial plexus nerves, liver, kidney, and spleen. Peripheral nerves were pinned to cards prior to fixation.

Light Microscopy and Autometallography

Tissues were processed routinely and embedded in paraffin. Formalin-fixed brains were cut into 5 mm thick coronal sections to allow visualization of various brain regions,

then each section was processed and embedded as the other tissues. Due to the presence of scleral ossicles, the eye was decalcified using the formic acid/sodium citrate method (Luna, 1968). Eyes were decalcified for 12 hours then processed and embedded routinely. All tissues for light microscopy were sectioned at 6 μm thickness and stained with Hematoxylin and Eosin (H&E).

Selected paraffin-embedded sections were stained with a metal-localizing stain using the silver amplification autometallography technique described by Danscher and Møller-Madsen (1985). An emulsion was made from four stock solutions. A 100 ml volume of the emulsion included 60 ml of 50% gum arabic, 10 ml of citrate buffer (25.5g citric acid and 23.5 g sodium citrate in 100 ml DI water), 15 ml of reducing agent (0.85 g hydroquinone in 15 ml DI water), and 15 ml of silver lactate solution (0.11g silver lactate in 15 ml DI water). Fresh stock solutions and emulsion were used for each set of slides stained. Slides were deparaffinized and placed in emulsion-filled jars and allowed to react, in the dark, for 45 minutes in a 26°C water bath. Slides were rinsed with tap water for about 1 minute, exposed to 5% sodium thiosulfate for 12 minutes at room temperature, rinsed again, then counter-stained with toluidine blue and viewed under light microscopy. Identification of the neuroanatomical regions of the brain which accumulated silver granules was based on a brain atlas of the chick (Kuenzel and Masson, 1988).

Electron Microscopy

Three birds from each treatment group were selected for electron microscopic (EM) evaluation of retinal structures. A wedge from the medial aspect of the right retina was fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, then embedded in epon-

araldite. One micron sections from selected areas were mounted on glass slides, stained with 1.0% toluidine blue, and viewed light microscopically. Blocks were further trimmed and sectioned by ultramicrotomy. Ultrathin sections (60-80 nm thick) were placed on copper grids, then stained with lead citrate and uranyl acetate and viewed on a Hitachi 7000 transmission electron microscope at the University of Florida Ophthalmology Electron Microscopy Laboratory.

Results

Gross pathology was unremarkable for most birds, and no treatment-related lesions were found. Incidental findings that may have affected results of other tests were found in several birds. In group A, bird Y-01 had intrathoracic fungal granulomas. In group B, bird O-17 had multiple findings including severe chronic arthritis of the scapulohumeral joints and scoliosis and malformations in the cervical and thoracic vertebrae. In group C, O-12 had swelling of the cranial tibial and long digital extensor tendons. Culture of the lesions would have been useful but was not performed at the time.

Organ weights and weights as percent of body mass are summarized in Tables 6.1 and 6.2, respectively. At a significance level of 0.05 there was no difference between group A (control) and treatments B or C for any tissue measurements as percent body weight, or as raw weights. Pectoral muscle percent (including superficial and deep pectoral) were slightly lower in females of group B than in females of group C, but this was significant only on the right side ($p = 0.0286$ and 0.0922 for right and left sides, respectively). Due to the asymmetry of this difference, as well as the lack of differences when compared to controls, it was considered a spurious result.

Ocular Tissues

The general ocular morphology of the cormorant eye is similar to that described for other avian species (Sillman, 1973; Meyer, 1986; Prince, 1956). The retina consists of the same layers typical of all vertebrate retinae (Figure 6.1). Below is a brief description of the appearance of the retina at the light microscopic level, followed by a comparison of findings among the treatment groups at both the light and EM level.

The majority of receptors appeared to be cones, with scattered rods, although specific differentiation was difficult at the light microscopic level, since some rods can look like cones, and in many cases the retinal pigment epithelium (RPE) obscured the outer segments. Peripherally there were fewer rods than centrally. The majority of photoreceptors also contained oil droplets at the base of the inner segments, which further indicated that most photoreceptors were cones. The outer limiting membrane (OLM), at the margin of the outer nuclear layer (ONL), was usually quite prominent. The ONL, composed of round to slightly oval nuclei of the photoreceptors, was 2-3 cell layers thick centrally, but thinned to 1-2 layers in the periphery.

The outer plexiform layer (OPL) was about the same thickness as the ONL. With H&E staining, the OPL appeared as a uniformly thick, acellular, dark pink-orange layer, usually with prominent pink fibers crisscrossing it between the inner nuclear layer (INL) and the ONL (presumably Müller cell processes). When viewed by EM, much of the OPL contained large pedicles interspersed with small spherules. In some areas there seemed to be few pedicles and many medium sized spherules. Rarely an area could be seen where there were very few of either type of process, and wide Müller cell processes could be seen spanning the OPL.

Table 6.1 : Organ weights (g) at necropsy, following 8 weeks of dosing with methylmercury chloride in the diet. Treatment A, B, and C are controls, high- (3.5 ppm), and low- (0.5 ppm) dose groups, respectively. L and R are left and right, respectively.

Tissue	Tx	n	Avg	Std	Min	Max
EYE (L)	A	7	2.62	0.19	2.31	2.87
	B	8	2.29	0.18	1.99	2.55
	C	7	2.56	0.30	2.07	2.93
EYE (R)	A	5	2.72	0.22	2.31	2.89
	B	6	2.51	0.64	2.04	3.91
	C	7	2.64	0.34	2.17	3.11
LIVER	A	5	40.73	4.39	33.03	46.63
	B	8	38.92	6.50	29.43	51.17
	C	7	35.60	5.99	29.76	45.86
SPLEEN	A	7	4.15	1.61	2.49	7.83
	B	8	2.84	1.23	1.16	5.05
	C	7	2.89	1.66	1.51	6.81
HEART	A	6	15.76	3.41	12.11	21.76
	B	8	14.84	2.88	12.41	21.51
	C	7	14.84	2.53	12.09	19.26
KIDNEY	A	7	18.49	1.89	15.68	21.02
	B	8	17.05	1.41	14.35	19.43
	C	7	16.37	1.54	14.77	18.45
LUNG-L	A	6	10.86	2.01	8.10	14.66
	B	8	9.50	2.29	7.33	14.53
	C	5	10.05	1.56	7.55	11.86
LUNG-R	A	6	11.01	1.93	7.67	13.23
	B	7	9.31	2.49	6.84	14.53
	C	5	10.93	2.65	7.13	14.49
BRAIN	A	7	9.76	0.50	9.14	10.55
	B	8	9.31	0.50	8.81	10.46
	C	7	10.03	0.77	8.81	11.17
SUP/DEEP PECTORAL-L	A	5	93.40	10.29	75.83	106.45
	B	6	79.59	8.03	68.76	92.87
	C	7	91.22	8.38	80.75	106.40
SUP/DEEP PECTORAL-R	A	7	90.60	10.24	74.78	105.59
	B	6	78.37	8.82	63.73	91.12
	C	7	92.94	8.03	83.29	107.20

Table 6.2 : Organ weights as percent of body weight, after 8 weeks of dosing with methylmercury chloride in the diet. Treatment A, B, and C are controls, high- (3.5 ppm), and low- (0.5 ppm) dose groups, respectively. L and R are left and right, respectively.

Tissue	Tx	n	Avg	Std	Min	Max
EYE (L)	A	7	0.17	0.01	0.16	0.18
	B	8	0.17	0.01	0.16	0.18
	C	7	0.18	0.01	0.17	0.19
EYE (R)	A	5	0.18	0.01	0.17	0.19
	B	6	0.18	0.04	0.15	0.26
	C	7	0.18	0.01	0.17	0.20
LIVER	A	5	2.72	0.16	2.49	2.92
	B	8	2.79	0.34	2.31	3.26
	C	7	2.48	0.15	2.14	2.66
SPLEEN	A	7	0.27	0.09	0.18	0.47
	B	8	0.20	0.08	0.09	0.33
	C	7	0.19	0.09	0.11	0.40
HEART	A	6	1.03	0.18	0.74	1.32
	B	8	1.07	0.17	0.82	1.42
	C	7	1.03	0.07	0.90	1.12
KIDNEY	A	7	1.22	0.04	1.16	1.29
	B	8	1.23	0.08	1.09	1.36
	C	7	1.15	0.06	1.07	1.24
LUNG-L	A	6	0.72	0.07	0.63	0.86
	B	8	0.68	0.13	0.52	0.93
	C	5	0.71	0.08	0.61	0.85
LUNG-R	A	6	0.73	0.07	0.60	0.82
	B	7	0.67	0.13	0.52	0.93
	C	5	0.76	0.11	0.58	0.88
BRAIN	A	7	0.65	0.04	0.59	0.72
	B	8	0.67	0.04	0.63	0.74
	C	7	0.71	0.05	0.63	0.78
PECTORAL-L	A	5	6.02	0.34	5.50	6.49
	B	6	5.93	0.31	5.53	6.35
	C	7	6.40	0.40	5.65	7.01
PECTORAL-R	A	7	5.97	0.34	5.29	6.48
	B	6	5.84	0.38	5.18	6.26
	C	7	6.53	0.42	5.74	7.00

The INL was quite thick with densely packed dark blue nuclei. It was usually between 2-4 times as thick as the ONL, depending on the location. Peripherally the entire retina was thinner than centrally, and the INL was also much thinner in this area. Occasionally displaced ganglion cells (GCs) could be seen in the inner portion of the INL. The inner plexiform layer (IPL) was by far the thickest layer in the cormorant retina. It generally stained a lighter pink (H&E) than the OPL and appeared as a meshwork of fine fibers.

There was a large degree of regional variation in the density and size of ganglion cells. Peripherally, there were very few GCs, most of which had small compact dark nuclei. Centrally, more medium and large GCs were visible and the density increased to the point of being 2-3 cell layers thick. In some areas, especially dorso-laterally, there were also very small, dark, round to oblong nuclei within the nerve fiber layer (NFL). Nerve fibers in this layer, in cormorants, are myelinated, and the nuclei found in the NFL were probably oligodendrocytes.

At the light microscopic level several differences were seen in the retinae of the high-dose group when compared to controls and low dose, but these were not always consistent or of uniform distribution. Most of the high-dose group appeared to have slightly thinner OPL than controls, as well as small vacuoles (with multifocal irregular distribution) in the NFL and the OPL (Figure 6.1). The high-dose group also seemed to have fewer large GCs, and some GCs with pyknotic and occasionally fragmented nuclei. The retinae of the low-dose group appeared similar to the controls. The findings listed above tended to be much more pronounced in the periphery of the retina, in particular the dorso-lateral region, and of varying severity among individuals.

The differences seen in the high-dose group by LM could not be substantiated when sections from selected birds were view by EM. The sections for EM were not taken from the same area as the LM samples, which may have contributed to this discrepancy. No vacuoles could be seen in the OPL or the NFL in the areas examined, and GCs in the high-dose group did not appear significantly different from controls. The main difference between the high-dose group and controls was observed in the photoreceptor outer segments. Many of the outer segments, especially of cones, of group B birds were swollen, with degeneration and fragmentation of disc lamellae (Figure 6.2). In the control and low-dose birds, small cones would also sometimes have irregular clumps of discs which did not fill the entire outer segment; however, they generally did not have the obvious swelling and fragmentation as seen in the outer segments of the B group. No distinct differences could be detected in the ONL, OPL or INL in either the high- or low-dose groups.

In both the low- and high- dose groups, changes were noted in the IPL, although alterations in appearance were different in each group (Figure 6.3). The low-dose group appeared to have disorganized and swollen synaptic processes, while the high-dose group had a generalized loss of detail, indistinct synaptic cell processes, and pronounced Müller cell extensions through the IPL. Ganglion cells in the low-dose group, when viewed by EM, had cytoplasmic vacuoles, which were apparent as a result of swollen endoplasmic reticulum (Figure 6.4). All three low-dose birds examined by EM had these vacuoles in the majority of their GCs, while the control and high-dose groups did not, except only rarely.

Staining for Hg, using autometallography, in ocular tissues resulted in deposits of very fine granules in several areas of the retina including the OPL, IPL, and the NFL

(Figure 6.5). The ILM generally stained very darkly in the high-dose group, making identification of individual granules difficult. In the low-dose group the ILM staining was much lighter. Photoreceptors also had Hg granules, but this was somewhat variable in intensity. The cell bodies on the retinal layers did not seem to accumulate mercury to the same extent that cells in other tissues did, rather, cell processes in the plexiform and nerve fiber layers seemed to be the preferential sites. Additionally, the granules tended to be relatively very small. The distribution of the granules in the retina was consistent with deposition in Müller cells, which span the entire retina, although this observation could not be determined unequivocally at the LM level.

The non-pigmented epithelium of the ciliary body also accumulated mercury granules in the high-dose birds (Figure 6.6). No granules were seen in this area in controls or in the low-dose birds. No lesions or mercury granules were found in other ocular structures such as the cornea, iris or lens.

Brain

Differences between the high-dose group and controls were generally subtle and often not consistent among all treated birds or in all brain regions. Throughout the cerebrum of high-dose birds there was a mild, diffuse gliosis, that mainly involved astrocytes. Several of the low-dose birds also appeared to have increased numbers of glial cells, but to a lesser degree than that in the high-dose group. The optic nerve and chiasm in high-dose birds also had an increased cellularity including oligodendrocytes and astrocytes, but no apparent differences otherwise. In several locations, mainly in the

midbrain and brainstem regions, some of the large neurons appeared slightly swollen. In the high-dose group, ependymal cells that lined the ventricles and the choroid plexus were also swollen. This finding was also seen in some of the low-dose birds, but in those instances the lesions were mild, having affected the ependymal cells irregularly. Compared to controls and low-dose birds, the high-dose birds seemed to have a large number of small, darkly-stained Purkinje cells in the cerebellum, and mild, focal gliosis of irregular distribution around the Purkinje cells. The granular cells did not appear to be affected.

In several instances, throughout the brain, there were areas where a few cells appeared as if they had early degenerative changes, including small cell size, dark eosinophilic cytoplasm, and eccentric small dark nuclei. However, similar appearing neurons were found in the same brain regions in the control birds; thus, little significance was attributed to this in terms of MeHg toxicity. Likewise, several birds from each group, including controls, also possessed a vacuolated appearance of several nerve fiber tracts within the brain, but a consistent association with MeHg could not be made. Perivascular cuffing was seen in several sections, but was not associated with any specific treatment.

Hg Distribution in Brain by Autometallography

In the high dose group there was diffuse staining of mercury in all areas of the brain, but large neurons tended to accumulate the most. In the rostral sections of the cerebrum, there was a fairly uniform light staining, which appeared as a fine dark cytoplasmic granularity of neuron cell bodies and of glial cells. In the forebrain regions, there were no specific areas that tended to have more mercury except for the large

neurons in the ectostriatum which had slightly more intense staining for Hg than the surrounding regions. An area corresponding to the nucleus rotundus had moderate to heavy staining for Hg, and in some birds the granules in this area seemed to be concentrated in the perinuclear region of the cytoplasm rather than diffusely throughout the cytoplasm as in other regions described.

The neuropil had very light, diffuse staining for mercury throughout all regions of the brain, including the cerebrum, cerebellum, midbrain (including the optic tectum), and medulla. Mercury granules in the neuropil appeared to be arranged in short linear patterns, and was likely within the axons and cell processes. Ependymal cells in the choroid plexus and lining the ventricles also stained heavily for Hg, and granules were found mainly in the cytoplasm toward the ventricular margin of the cells.

The optic nerve and other myelinated tracts within the brain stained diffusely light brown in all treatments so localization of Hg could not be assessed accurately in these areas. However, in the high-dose group these tracts did stain more intensely than the controls or low-dose group. Clear and distinct granules, as seen in the cytoplasm of neuron cell bodies, were not usually apparent in the fiber tracts.

In the more caudal regions of the brain, i.e., in the midbrain, cerebellum, and medulla, the accumulation of mercury granules was more variable and often more intense than in the cerebrum. Most neurons and glial cells had at least some visible granules. Several areas had significantly more intense staining than others. In the cerebellum, Purkinje cells were moderately to heavily stained, and the granular cells were lightly to moderately stained diffusely (Figure 6.7). Several areas of the optic tectum and midbrain

stained heavily, including large neurons in the region corresponding to the magnocellular nucleus or Imc (Nucleus isthmi pars magnocellularis) (Figure 6.8), cells of the stratum griseum centralle (SGC), and the cells lining the ventricle of the optic tectum (ventriculus tecti mesencephali). Of all brain areas examined, the Imc was the most heavily stained for mercury, having blackened cell bodies which were visible even at low magnification. Interestingly, the area corresponding to the Ipc (Nucleus isthmi pars parvocellularis), adjacent to the Imc, had relatively moderate accumulation of mercury granules (Figure 6.8). Other midbrain areas with moderate to heavy mercury staining included the nucleus ruber (RU) and the nucleus mesencephalicus lateralis pars dorsalis (MLD). The majority of other nuclei in the midbrain and medulla stained very lightly to lightly, and only occasional nuclei were lightly to moderately positive for Hg. In the caudal region of the medulla there was light to moderate staining in the lateral reticular nucleus, and moderate staining in the ependymal lining of the spinal canal and in a nucleus corresponding to CN XII. All other nuclei in the posterior medulla had very little reaction for Hg.The cranial cervical spinal cord was very lightly stained for mercury. More caudal spinal cord segments were not evaluated for Hg localization.

In the low-dose group there was diffuse, very light Hg staining in some areas of the neuropil and only occasional cell bodies were seen with any visible granules. In the low dose group very fine granules were also seen in ependymal cell cytoplasm (only visible at 1000x). No Hg granules could be seen in neurons, neuropil, or support cells in the brains of control birds.

Spinal Cord and Peripheral Nerves

In the high-dose group, the cranial and caudal cervical spinal cord had an increased number of myelin sheaths with missing axons. As a result of the missing axons the dorsomedial tracts in most high-dose birds had a diffusely vacuolated appearance. Other areas with noticeable missing axons included the dorsolateral and lateral aspects of the spinal cord. In several high dose birds, the myelin sheaths of the dorsolateral tracts in particular had a disrupted or fragmented appearance. Several birds also had a few degenerating and necrotic neurons in both the dorsal and ventral horns. As with other lesions described, the severity and distribution were not uniform and varied among birds within the high-dose group.

Sciatic nerves in the high-dose group had swollen myelin sheaths containing eosinophylic debris and Schwann cell hyperplasia (Figure 6.9) was present. Some controls and low- dose birds also possessed areas with apparent myelin swelling, so this may have been an artifact of tissue processing. The main characteristic which distinguished the high-dose from either the controls or the low-dose birds was the presence of numerous digestion chambers with eosinophilic debris (Wallerian degeneration). Although a few control and low-dose birds had mild lymphocytic infiltrates around the nerves, the number and severity of this condition in the high-dose group was much greater. Several of the high-dose birds had intraneural pericapillary lymphocytic infiltrates in addition to the perineural infiltrates. Brachial nerves from several individuals from each group were examined, but they did not have the changes that the sciatic nerves possessed. Except for rare digestion chambers found in the high-dose birds, the appearance of brachial nerves was not appreciably different among the groups.

Other Tissues

When examined with routine H&E staining of histological preparations, MeHg-treated groups did not have any specific liver lesions which could be attributed to mercury toxicity. An incidental finding in the livers was focal diffuse mixed inflammatory infiltrates, usually with central areas of necrotic hepatocytes. In most instances these lesions were found periportally, but at times they were seen in midzonal or central lobular regions. Occasionally, biliary hyperplasia and lymphoid necrosis were also seen with these lesions. The severity of this condition was variable among birds, and this type of lesion was present in all birds from all treatment groups.

Mercury localization stain in the liver resulted in abundant granules deposited along the canalicular margin in the hepatocytes of both the low- and high-dose groups (Figure 6.10). Staining and granule deposition was uniform and diffuse throughout the sections, and seemed more intense in the high-dose group than the low-dose group. No granules were seen in hepatocytes of the control birds.

Kidney and spleen also did not have any lesions that could clearly be associated with MeHg. An incidental finding was the presence of gravid flukes in the kidneys of several birds, and variable lymphocytic infiltrates. Vascular prominence was seen in the spleen of several birds from control and high-dose groups and could not be linked to treatment.

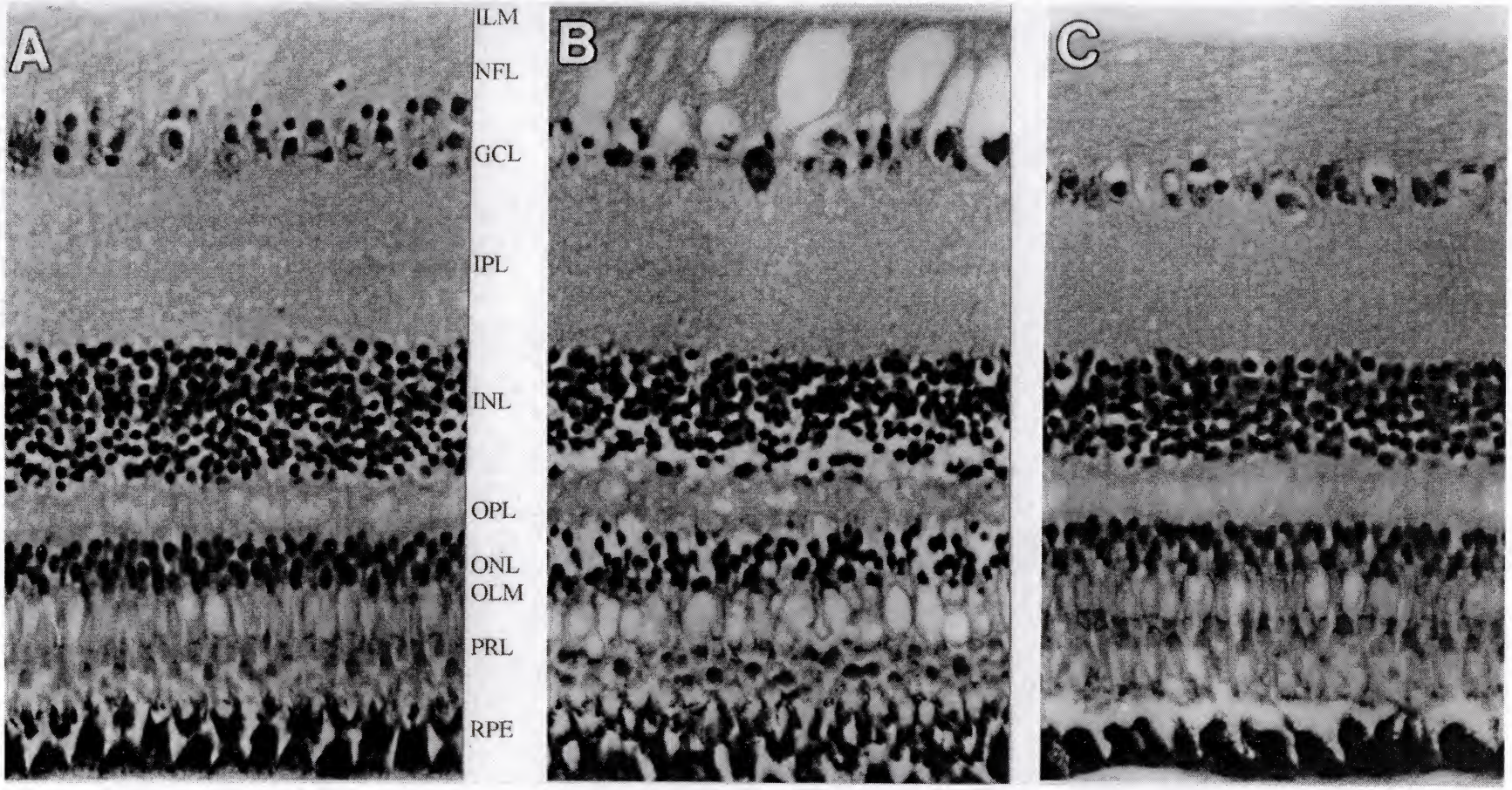


Figure 6.1: Retina of A - controls, B - high-dose, C - low-dose MeHg groups. Sections are from a midperipheral area of the dorsolateral region of the retina. Note vacuoles in NFL and disrupted OPL with small vacuoles in B. The ONL and inner segments of the PRL also appear different in B, but this was much less consistent than alterations in the NFL and OPL. Several areas in control ONL and PRL also had this appearance, so it was most likely not related to MeHg. RPE, retinal pigment epithelium; PRL photoreceptor layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL inner nuclear layer; IPL, inner plexiform layer; GCL ganglion cell layer; NFL, nerve fiber layer; ILM, inner limiting membrane. H&E, 400x.

Figure 6.2: Electron micrograph of photoreceptor outer segments from the retinae of A - control, B - high-dose, C - low-dose MeHg groups. Note swelling of the segment and disc lamellae fragmentation in group B. 10,000x.

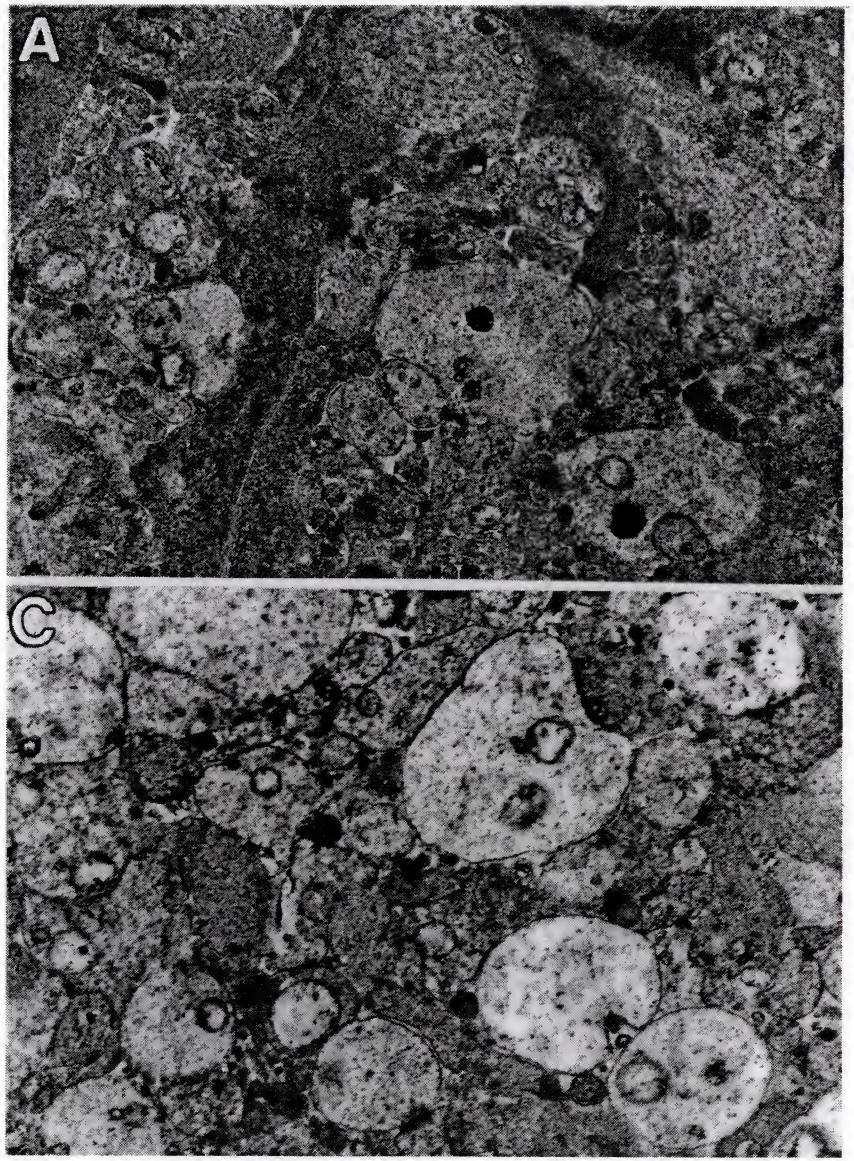


Figure 6.3: Electron micrograph of inner plexiform layer from the retinae of A - control, B - high-dose, C - low-dose MeHg groups. Note prominent Müller cell processes (arrow heads) and general lack of contrast and detail in B. Section C shows disorganized morphology and swollen synaptic processes. 10,000x.

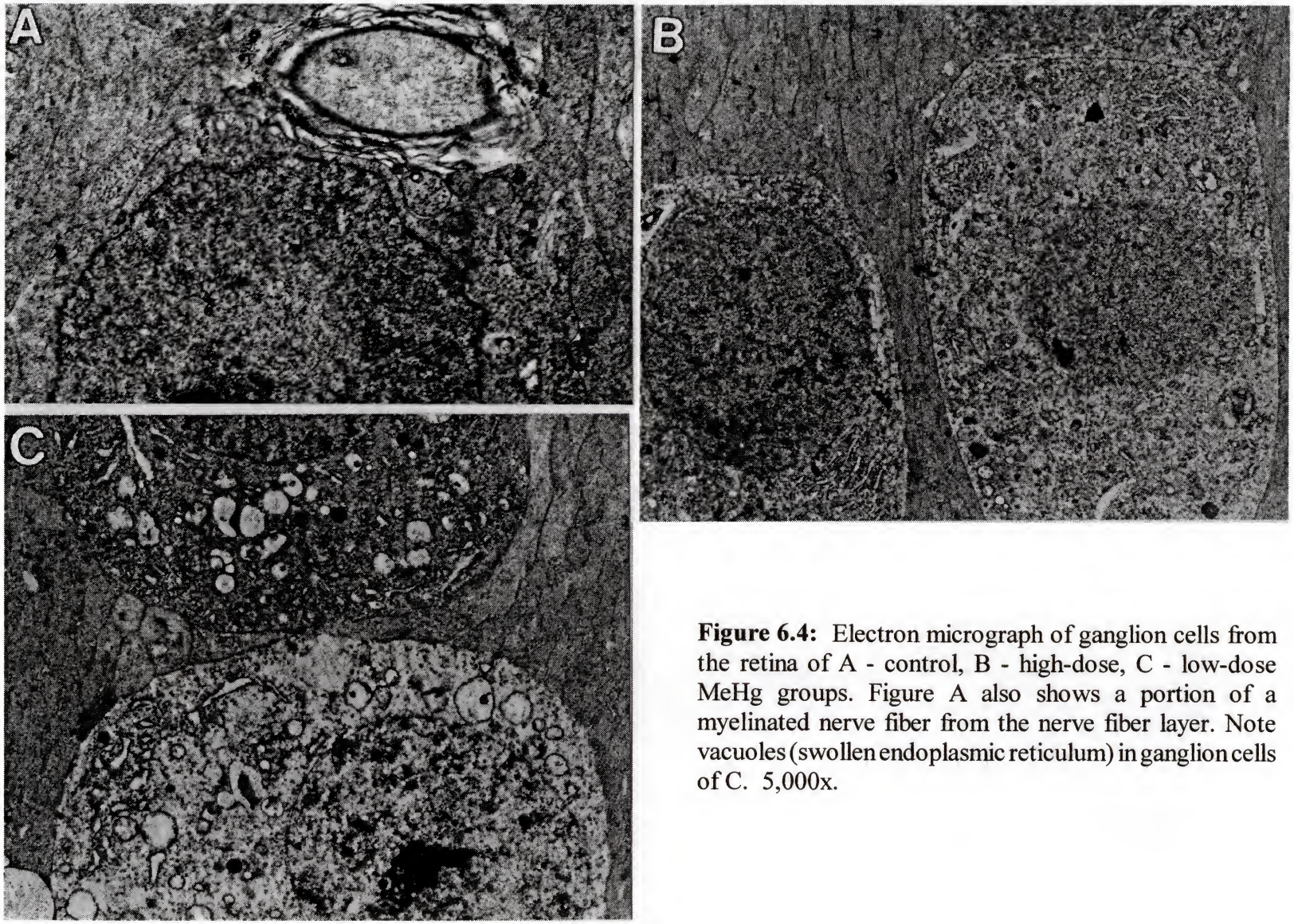


Figure 6.4: Electron micrograph of ganglion cells from the retina of A - control, B - high-dose, C - low-dose MeHg groups. Figure A also shows a portion of a myelinated nerve fiber from the nerve fiber layer. Note vacuoles (swollen endoplasmic reticulum) in ganglion cells of C. 5,000x.

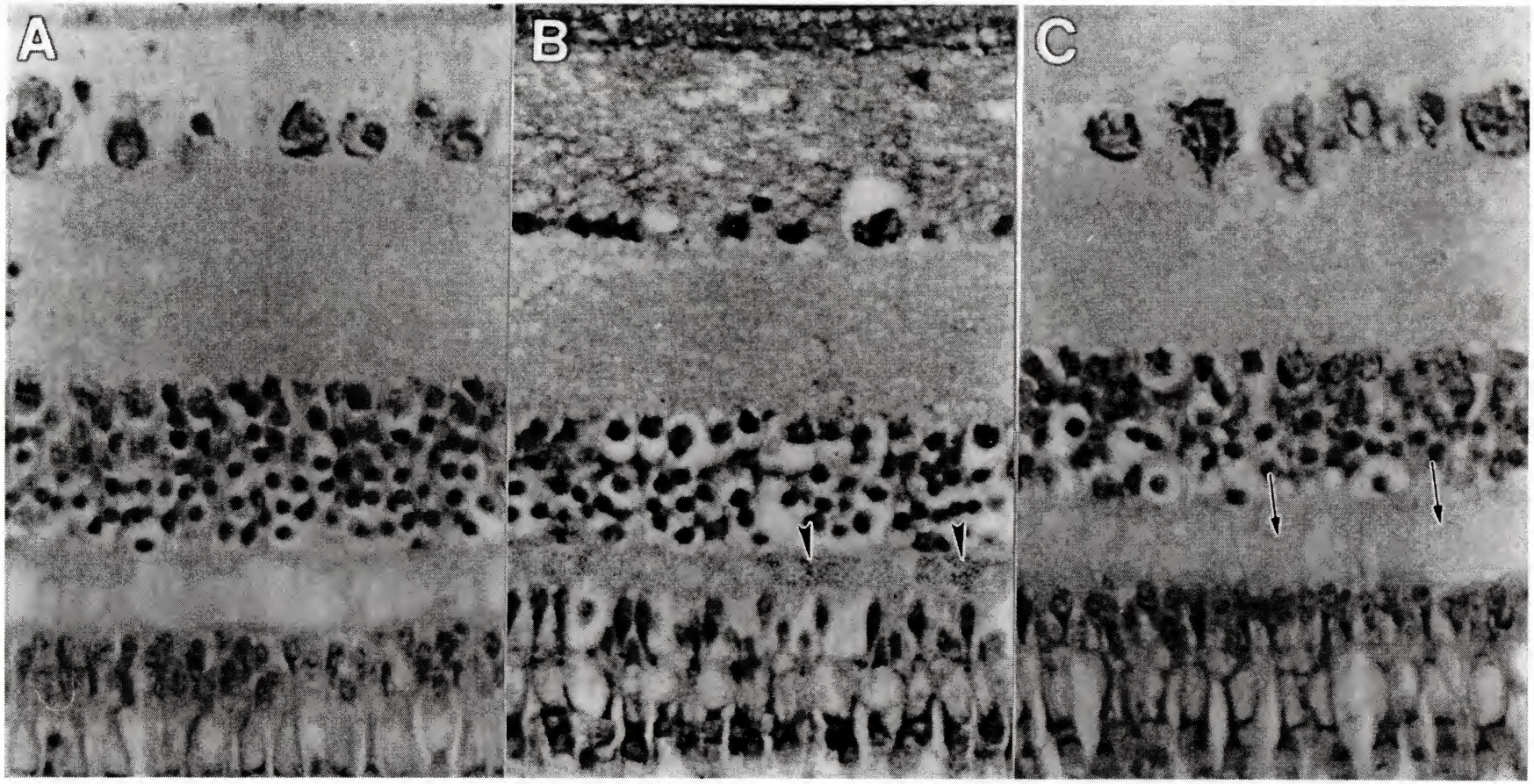


Figure 6.5: Mercury localization in retina using autometallography. A - control, B - high-dose, C - low-dose MeHg groups. Note dark granules in OPL (arrowheads) of B. Granules are silver which deposits as a result of the presence of mercury. In B fine granules can also be seen diffusely in the IPL, NFL, and the PRL. Individual granules are difficult to discern in the ILM, but the entire layer stains very darkly compared to A and C. In C very faint granules can be seen in the OPL (arrows). The different thickness of the various layers is mainly a result of regional differences which occur normally. 640x.

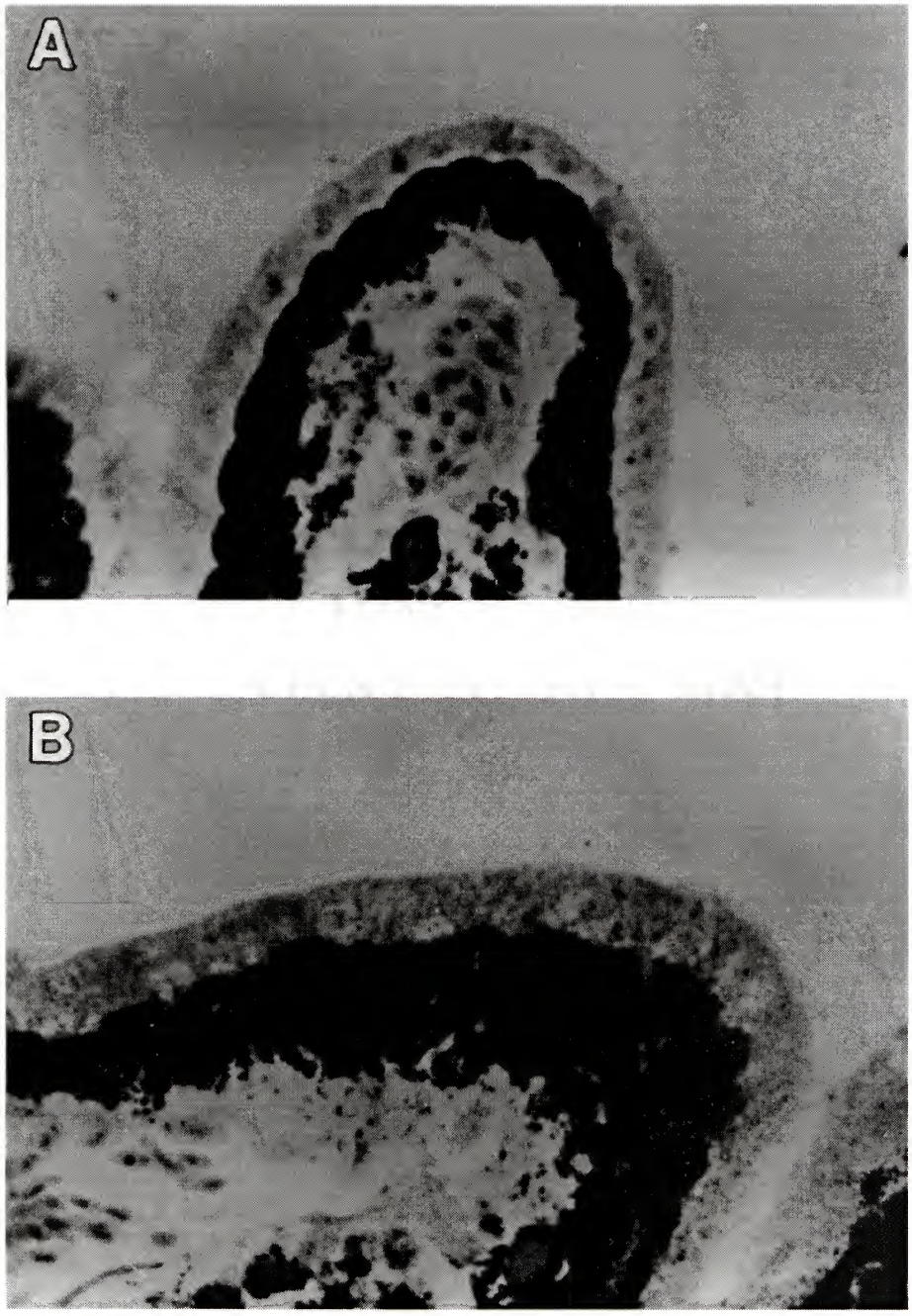


Figure 6.6: Mercury localization in the ciliary epithelium. A - control, and B - high-dose MeHg groups. Numerous fine granules were visible in the non-pigmented ciliary epithelium. No granules were visible in the low-dose group. 640x.

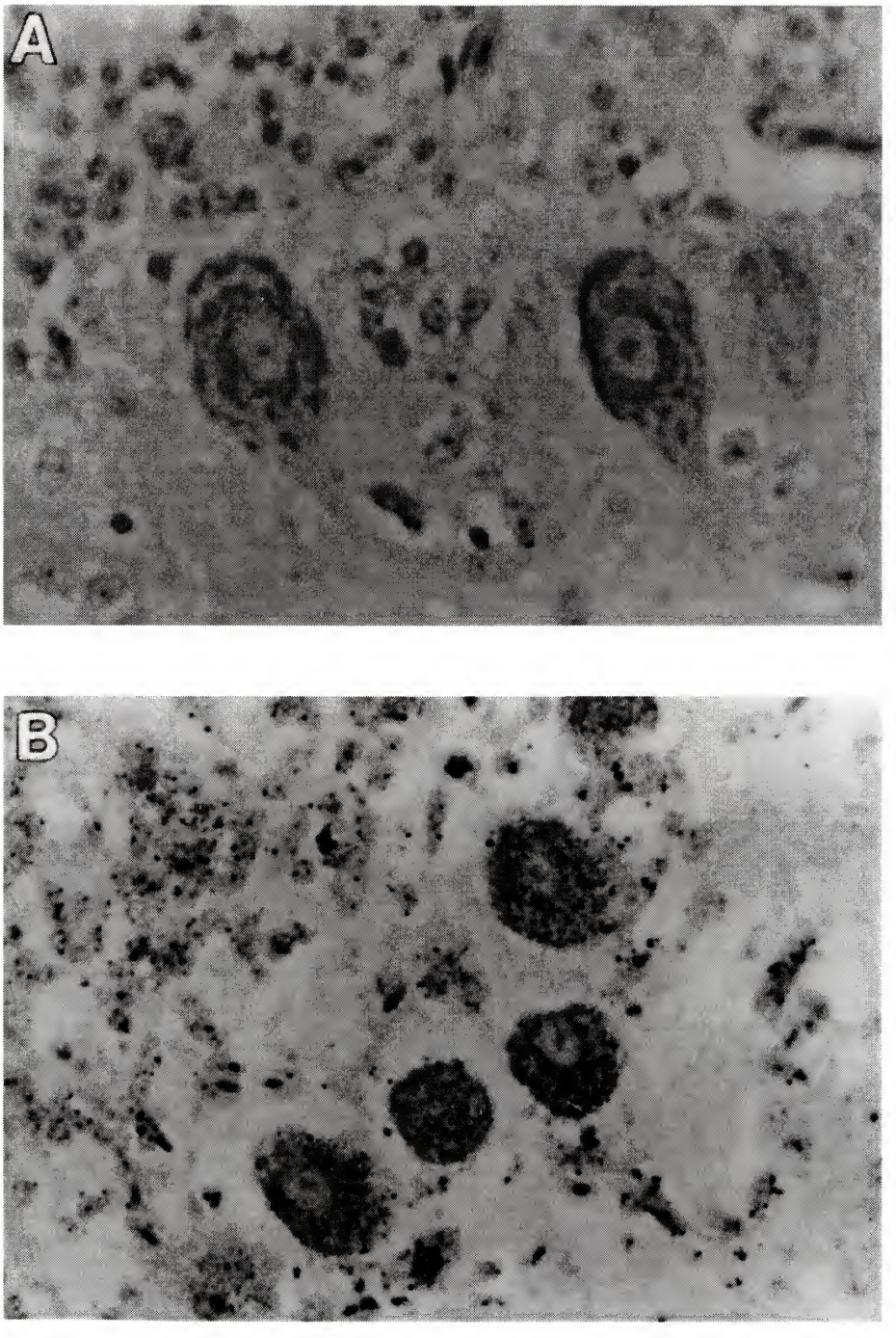


Figure 6.7: Mercury localization in the Purkinje cells in the cerebellum. A - control, and B - high-dose MeHg groups. Note numerous dark granules in cytoplasm of Purkinje cells in B. Granular cells in upper left of B also contain granules. In comparison, controls were free of granules. No granules were visible in similar sections in the low-dose group. 640x.

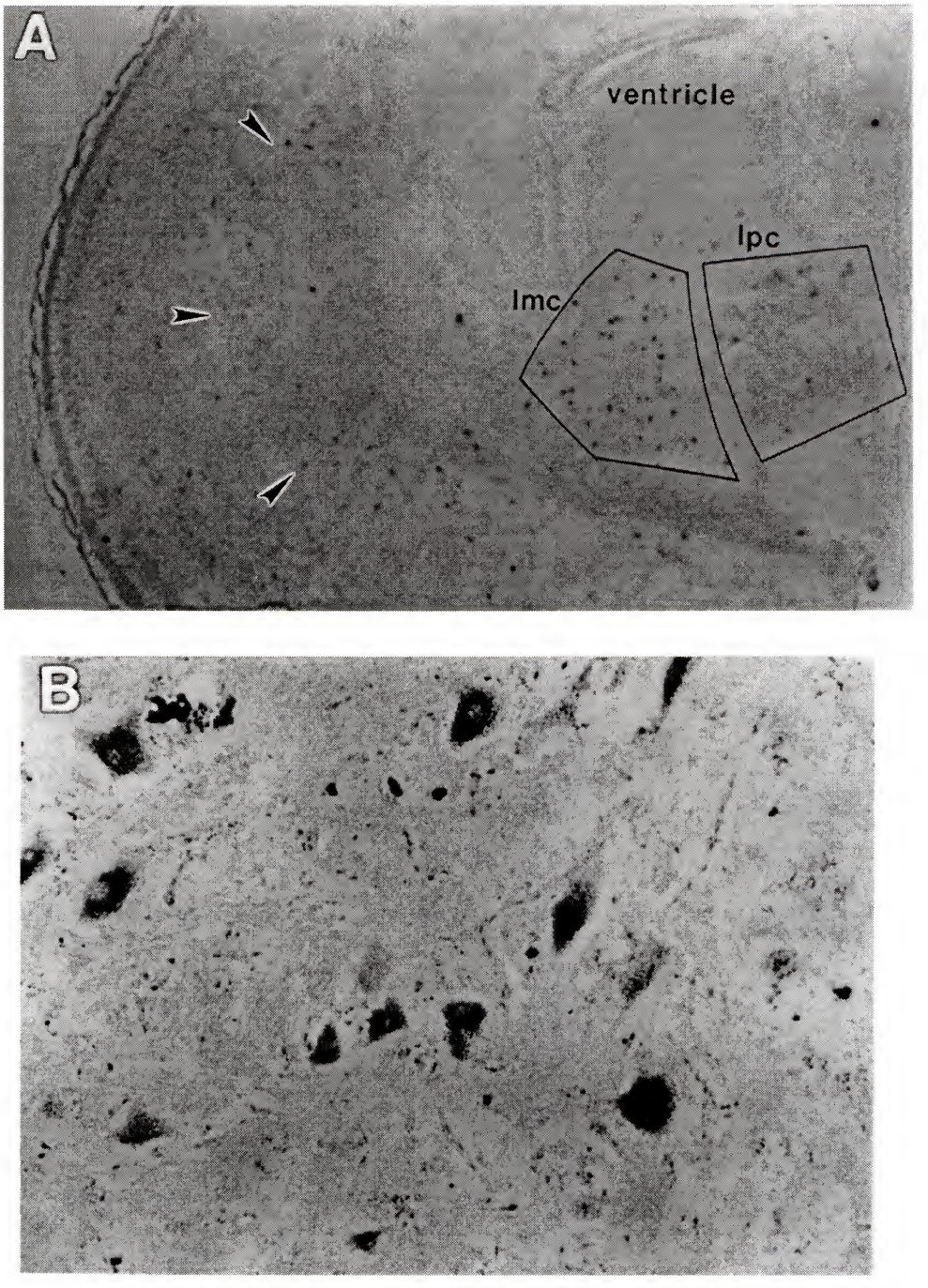


Figure 6.8: Mercury localization in the optic tectum at 20x (A) and 200x (B) in a high-dose bird. In A, nuclei corresponding to the Imc (nucleus isthmi pars magnocellularis) and Ipc (nucleus isthmi pars parvocellularis) are indicated. Note that the Imc region stained much more heavily than surrounding areas. The SGC layer of the optic tectum (arrowheads) and the Ipc had moderate to heavy staining for mercury. At higher magnification of a region of the Imc (B) it can be seen that granules completely fill the neurons and obscure cellular detail.

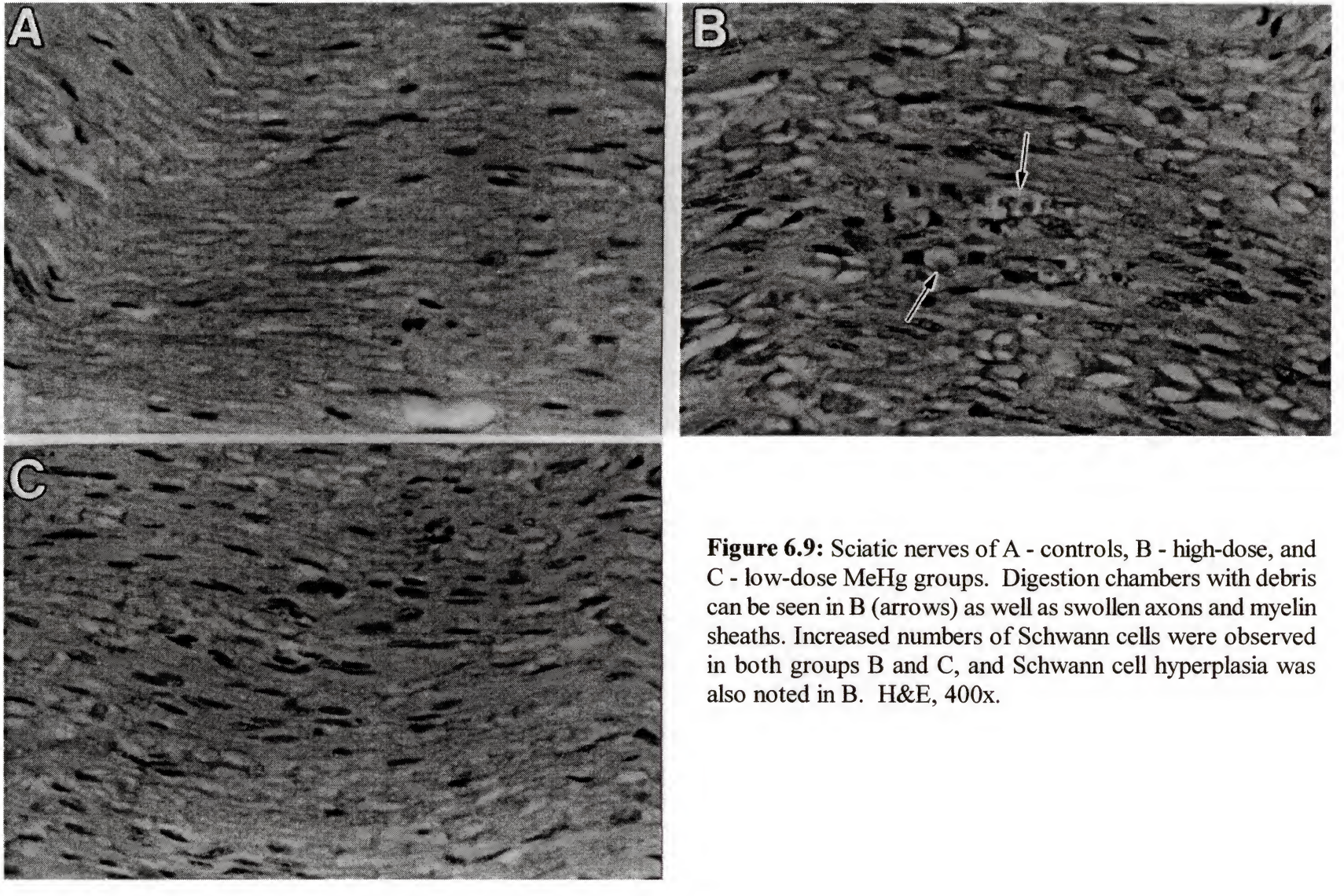


Figure 6.9: Sciatic nerves of A - controls, B - high-dose, and C - low-dose MeHg groups. Digestion chambers with debris can be seen in B (arrows) as well as swollen axons and myelin sheaths. Increased numbers of Schwann cells were observed in both groups B and C, and Schwann cell hyperplasia was also noted in B. H&E, 400x.

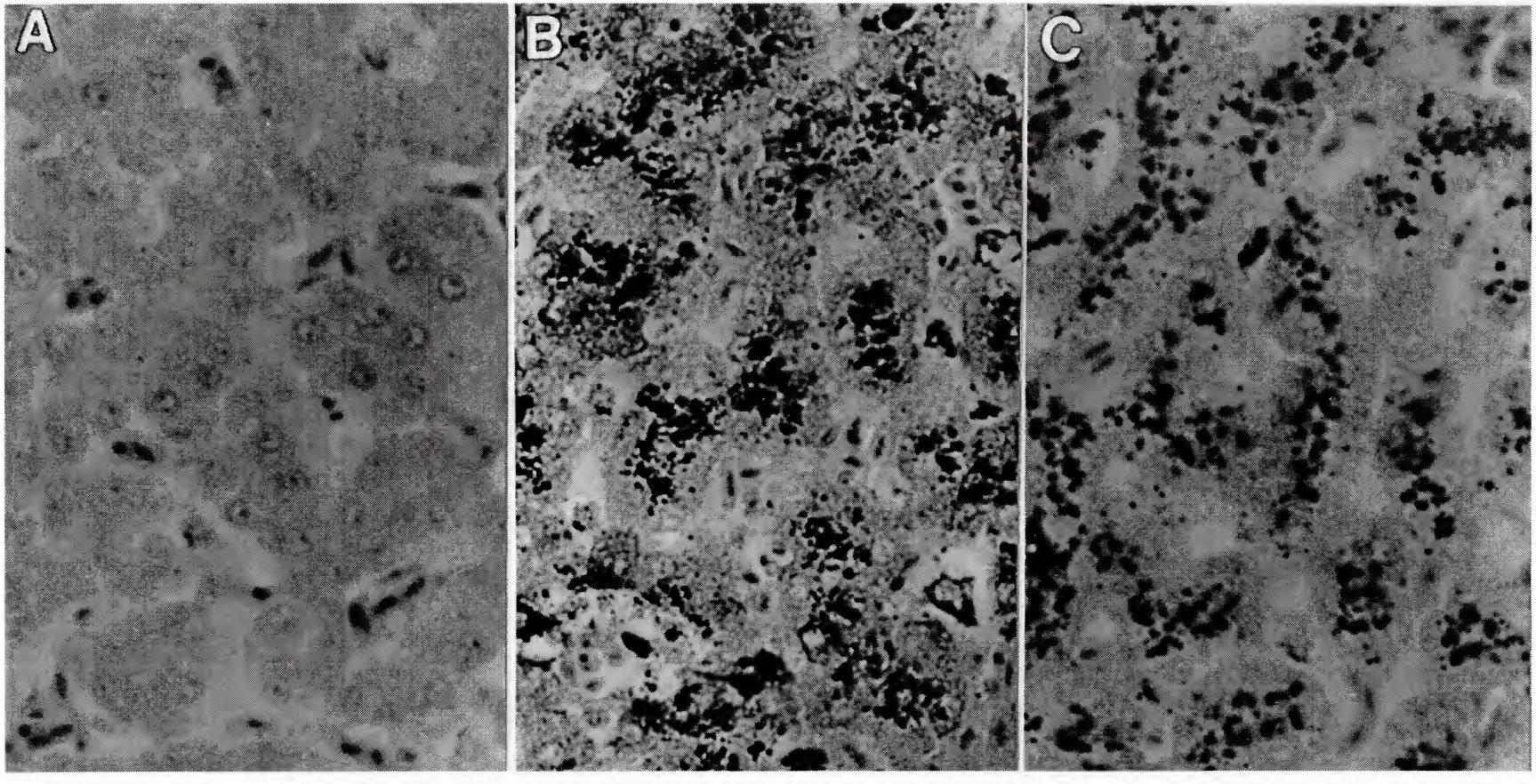


Figure 6.10: Mercury localization in liver using autometallography. A - controls, B - high-dose, C - low-dose MeHg groups. Granules in the liver are much larger than those in the brain. Granules seem to accumulate preferentially in the canalicular margin of the hepatocytes. 640x.

Discussion

The main neurological findings in the high-dose group included a Wallerian-like degeneration, especially of the sciatic nerve, axonal degeneration in the cervical spinal cord, and swollen ependymal cells in the brain. Other, less pronounced findings included occasional neuronal degeneration in the spinal cord and a diffuse gliosis throughout the brain, especially in the cerebrum. Lesions in peripheral nerves in the cormorants were very similar to those described in MeHg poisonings of mallard ducks (Pass et al., 1975), red-tailed hawks (Fimreite and Karstad, 1971), and goshawks (Borg et al., 1970). Pass et al. (1975) also described a similar, but much more severe, axonal degeneration in the cervical spinal cord compared to that found in the cormorants. Demyelination of spinal cord segments and peripheral nerves was not distinctly observed in the cormorants when sections were viewed by routine H&E staining. A myelin stain such as Luxol-Fast-Blue would be needed to verify if perhaps a mild demyelination was present which was not obvious when viewed by H&E.

Peripheral nerve and spinal cord lesions were likely a major contributing factor to the proprioceptive deficits detected on neurological exams (Chapter 8). Although terminology and specific anatomical components of the spinal cord in birds vary somewhat from mammals, it is thought that dorsal and dorsolateral spinal regions are involved in the proprioceptive mechanism. The dorsal funiculus contains fibers carrying incoming sensory impulses corresponding to the cuneate and gracilis, and dorsolaterally there is an area corresponding to the spinocerebellar tract in mammals. Descending motor components are thought to involve a cerebellospinal tract, medial to the spinocerebellar tract, and

tecteospinal and rubrospinal tracts found in the lateral funiculus (Benzo, 1986; Webster et al., 1990).

Given the presence of proprioceptive deficits and lesions in the sciatic nerves, it was somewhat surprising that more pronounced neuronal degeneration was not apparent in the spinal cord or brain. Although a few degenerating and necrotic neurons were seen in the spinal cord, this lesion was not as consistent among treated birds as was the peripheral nerve effect. It is possible, however, that more damaged neurons may have been found in more distal spinal cord segments which were not examined. Interestingly, brachial nerve lesions in the cormorants were minimal, a finding which differs from the findings in mallards (Pass et al., 1975). The significance of this is uncertain, but one could speculate that perhaps it is related to axonal length. Long sensory and motor axons of the PNS or the long ascending sensory or descending motor axons of the CNS may potentially be more vulnerable to toxicants (Anthony et al., 1996).

Although a diffuse astrogliosis was seen in the brains of high-dose birds, specific cell counts would be needed to verify and quantitate this. Both generalized and diffuse gliosis in the CNS have been reported in MeHg toxicity in other birds (Borg et al., 1970; Pass et al., 1975), as well as for other species (Charleston et al., 1994; Shaw et al., 1975; Tryphonas et al., 1973; Charbonneau et al., 1974). Gliosis usually occurs along with neuronal necrosis, but it has also been shown to be an early change, prior to neuronal loss, in chronic subclinical methylmercury exposure (Charleston et al., 1994).

While no unequivocal neuronal degeneration was found in the forebrain regions of the MeHg treated cormorants of this study, this is not entirely surprising. There is a great

deal of variability in species sensitivity to MeHg, as well as variability in the dosing rates used in previous studies, which makes comparisons difficult. In the majority of avian studies, high doses at short intervals were required to produce clinical neurotoxicity and CNS lesions.

In a study on mallards, 13.2 mg of MeHgCl was given by day 17 of dosing and resulted in brain levels of 13.5 ± 6.0 µg/g of mercury and the presence of both central and peripheral nervous system lesions (Pass et al., 1975). In the present study the cormorants in the high-dose group received a total of about 50 mg total MeHgCl over 56 days. At 14 days, the cormorants in this group had received approximately 13 mg of total mercury, which is comparable to the mallard study. The principal difference in dosing between the studies was that the cormorants received the total amount of mercury in smaller doses at more frequent intervals, compared to larger doses at 3-4 day intervals in the mallards. These types of differences may play an important role in the systemic distribution of Hg. While mallards had 13.5 ± 6.0 µg/g brain Hg after 2 ½ weeks of dosing, the brains of cormorants only contained 11± 1.7 µg/g after 8 weeks. The presence of CNS lesions may be related to the rate at which the brain accumulates Hg as well as to the final tissue concentration. The decreased VEP amplitudes detected as early as 4 weeks of dosing (Chapter 6), but without severe pathological changes, implies that functional neurologic impairment occurs prior to structural alterations. Also, it may be that ultrastructural alterations occur in the brain prior to changes recognized by LM.

The significance of swollen ependymal cells, which also stained heavily for Hg, is uncertain. Ependymal cells in mallards were also reportedly stained, but no pathology was mentioned in that study (Pass, 1975). Similarly, in rats dosed with 20 mg/ml in drinking

water ependymal cells stained for Hg at about day 20, which was at or just prior to onset of clinical signs (Møller-Madsen, 1991). Ependymal cells function in the formation of cerebrospinal fluid (CSF), and damage to these cells could alter the composition of the CSF with potentially widespread effects since the CSF contacts all parts of the CNS. This particular finding was viewed cautiously, since swollen ependymal cells have not been reported in birds or any other species in relation to MeHg.

In spite of scant pathological changes of the CNS, significant accumulations of mercury were visualized using autometallography. Mercury localization in the brains of cormorants at the 3.5 mg/kg daily dietary MeHg level had a selective distribution and variable intensity of staining in different regions. Identification of brain regions was based on the stereotaxic anatomy of the chick brain (Kuenzel and Masson, 1988). The reason for the selective accumulation of Hg, not just in specific brain regions, but in specific nuclei, is not entirely clear. Sensory systems tend to be sensitive to methylmercury (Komulainen, 1988), and it has been suggested that changes in axonal transport may play a role in the selective nature of Hg in the brain (Aschner et al., 1987). Variability in the biochemistry of different cells may also play a role in this phenomenon.

The autometallography techinque is based on the principle that silver ions can be reduced to metallic silver, by a developer, on the surface of metal sulfides and selenides (Danscher, 1981; Danscher and Møller-Madsen, 1985). Presumably, the more metal sulfide or selenide available, the more metallic silver will precipitate. The Imc, in the optic tectum, stained much more heavily than other regions of the brain, while another component of the isthmi complex, the Ipc, had appreciable staining but much less than the

Imc. The isthmi complex, which includes the Imc and Ipc, is involved in auditory processing and has also been shown to be involved in control of pupil size (Pearson, 1972). It is likely, however, that this complex is related to other functions as well. Stimulation of the Imc resulted in behavioral changes including crouching and running with sleeking of the feathers (Philips, 1964).

The SGC of the optic tectum, the nucleus rotundus, nucleus ruber or red nucleus, the MLD and cerebellar Purkinje cells also had relatively intense staining for Hg. The SGC is the source of efferent fibers to the nucleus rotundus, and both areas are important components of the tectofugal visual pathway in birds (Engelage and Bischof, 1993). The red nucleus is thought to be involved in locomotor control. The MLD is a major auditory center with axons from the trigeminal sensory nucleus and the vestibulo-cochlear area, and with some fibers relating to the lateral lemniscus, the isthmo-mesencephalic tract, and the optic tectum (Pearson, 1972). The cerebellum receives abundant sensory input and functions as the major motor control center, maintaining equilibrium and coordinating muscle movement. The function of Purkinje cells in particular involves inhibitory modulation of excitatory signals to deep nuclear cells which provide output signals from the cerebellum (Guyton and Hall, 1996).

Since little pathology was found in the brain, it was not possible to make an association between the presence of Hg and toxicity to cells. However, it has been shown, at least with regards to Purkinje cells, that these cells can be affected by MeHg in birds (Fimreite and Karstad, 1971), but at doses higher than those in the present study. Likewise, lesions to neurons in the midbrain and medulla, which in the cormorants also

had significant accumulation of Hg, have also been reported in some birds poisoned with MeHg (Borg et al., 1970; Heinze and Locke, 1976). In most avian studies peripheral nerve and spinal cord lesions were reported more commonly and seen more consistently than neuronal damage in the brain.

The areas which stained most heavily for Hg were primarily sensory centers, mainly auditory and visual, and the main sensory-motor integration center, the cerebellum. Pass (1975), who studied the distribution of methylmercury in the mallard duck using microautoradiography, found a tendency for specific localization of grains over certain cell types, most notably over the cytoplasm of astrocytes. One bird in that study also had concentration of silver granules over the cytoplasm of large neurons in the parasagittal columns in the spinal cord, pyramydal ventral horn neurons throughout the medullary nuclei, and cerebellar mesencephlic and diencephalic nuclei. Additionally, Pass (1975) noted that staining of the nucleus rotundus in that bird was very prominent. The mallard study utilized labeled MeHgCl for dosing, while in the cormorants Hg localization in tissues was conducted using autometallography involving a silver emulsion technique. The latter technique stains mercury which is bound as sulfides and selenides in the tissue, which means that any unbound mercury is most likely not stained. It has also been argued that the silver emulsion technique may only stain inorganic forms of Hg (Rodier et al., 1988). These potential limitations of autometallography, along with possible interspecies and dosing differences, may account for the differences between the two studies with regard to Hg localization in the CNS.

Ocular histopathology at the LM level in the high-dose group included vacuolation of the NFL and derangement of the OPL, especially peripherally. On EM, swollen

photoreceptor outer segments and fragmented disc lamellae were also apparent, and changes were noted in the IPL in group B and C, and in the GCs of group C. Mercury was localized in all layers but was most pronounced in the OPL, IPL, and NFL, and ILM.

Although effects on vision due to MeHg exposure are thought to be a result of centrally mediated effects, the presence of altered retinal morphology in the cormorants suggests that perhaps there may be a peripheral component. Very few studies have reported any retinal pathology as a result of mercury exposure. In isolated axolotl retina, mercuric chloride caused swelling and granulation of rod outer segments (Tessier-Lavigne et al., 1985). In rats treated with methylmercury, mercury was found in all retinal layers including Müller cells and the inner segments of photoreceptors, but not in the outer segments, and no pathology was reported (Gramoni, 1980). In both of the above studies electroretinographic changes were also noted. A study in great egrets dosed with 5 mg/kg daily MeHg resulted in an appearance of the retina at the light microscopic level similar to that seen in the cormorants (Loerzel et al., 1996). Photoreceptor lesions on EM, and Hg localization patterns in the retina and ciliary epithelium, were also similar in cormorants and egrets. Degenerating cells were also found in the outer nuclear layer in the egret study and ERG parameters were altered.

The retinal findings in the great egrets were originally viewed with skepticism since the results were base primarily on one dosed bird, the only one of four birds (2 dosed and 2 controls) to survive to the end of the study. In the present study the tendency has been to be conservative in the interpretation of the ocular lesions, since there is little evidence in any MeHg studies that the retina is susceptible to pathological damage from this toxicant.

Even in studies showing functional change at the retinal level, in *in vivo* ERG studies, retinal pathology has not been described. However, the striking similarity between the lesions seen in the egret and those seen in the high-dose group of cormorants, along with the comparisons to control cormorants, prevents the dismissal of these findings as entirely spurious or incidental findings.

If one is to accept that the lesions are indeed MeHg induced, several hypotheses can be considered. One, that the cormorant retina is relatively sensitive to MeHg toxicity, since total ocular mercury was quite low compared to other tissues which had no pathology. Average ocular Hg content in the high-dose group was only 2.5 ± 0.7 μg/g, while CNS level was 11±1.7 μg/g. Two, the ocular pathology was not severe enough to be detected by flash ERG analysis. This finding in itself is not necessarily unusual since sometimes large numbers of abnormal cells must be present to appreciably affect the ERG. Alternatively, if lesions have a regional distribution, as seems to be the case in the cormorants, then the flash ERG used in this study may not be adequately sensitive since it measures a composite response from the entire retina. Three, in cormorants MeHg may potentially have effects on vision, prior to overt neurotoxiciy, which at least in part may have a peripheral retinal component contributing to the deficits.

Further evaluation of additional birds and including other retinal areas at the EM level, would be useful in verifying a definitive relationship or the lesions to MeHg exposure. The sections taken for EM were from a central region of the medial to ventro-medial aspect of the retina, while the LM sections were from the lateral retina. This may account for some of the discrepancies between LM and EM findings. The lack of vacuoles

in the NFL and no apparent changes in the OPL, when viewing the retinae at EM level, could be related to the location in the retina. Another discrepancy in the results was that differences were seen in several retinal layers, on EM, in the low-dose birds. The low-dose level was not expected to result in any pathology, and Hg levels in the eyes of these birds was very low. Potentially, MeHg in very low doses could react differently, mechanistically, than at a higher dose, but this remains speculation at this point. Considering these findings, lesions should be interpreted cautiously. Further examination of other areas of the retina as well as examination of more birds at the EM level may be useful to determine the extent and consistency of these findings.

The most pronounced findings in the high-dose group, by light microscopy, were in the periphery of the dorsal aspect of the lateral retina, indicating a possible regional variation in the effects of MeHg within the retina. The avian retina is not homogeneous in terms of its cell characteristics and distribution. In pigeons, for example, the dorsolateral retina functions in binocular vision, has a larger proportion of red oil droplets (hence it is termed the red field) compared to yellow droplets which predominate in the remainder of the retina, and contains more amacrine synapses and a more complex IPL than other retinal regions (Nalbach et al., 1993). The eyes of cormorants are positioned slightly more frontally than pigeons, but it is likely that the dorsolateral aspect of the retina in these birds is also involved in binocular vision, as well as in visual processes related to food acquisition, and likely has cellular and perhaps biochemical differences from the other retinal regions. Direct comparison to the pigeon retina cannot be made due to considerable species variations and specific ecological adaptations. For this reason, without studies on

cormorants, the specific brain regions to which the dorsolateral retinal ganglion cells project cannot be determined. The dorsolateral retinal region in many birds of prey projects mainly to the lateral geniculate complex (thalmofugal visual pathway), while in pigeons only a few ganglion cells from this region send efferent axons to the geniculate complex (Güntürkün et al., 1993).

Liver, kidney, and splenic lesions were not observed, and were not expected at the doses and durations used in this study. Lesions in liver and kidney at these levels and duration of MeHg have not commonly been reported in birds, and have usually only been associated with much higher, overtly neurotoxic levels. Borg et al. (1970) found a slight acute nephrosis with fatty degeneration of the tubular epitherlium, and degeneration of proximal tubules, and fatty degeneration of Kupffer cells in goshawks fed 10 and 113 ppm MeHg for 4-5 weeks. Livers of Pekin ducks fed 15 ppm MeHg for 12 weeks showed an increase in lipid droplets and pericanalicular granules on light microscopic examination, as well as other ultrastructural changes (Bhatnagar et al., 1982). In the same study, ducks dosed with 5 ppm had a moderate increase in pericanalicular lysosomal bodies and more active Kupffer cells with larger phagocytic vacuoles. These types of lesions were not observed in the cormorants; however, Hg localization revealed deposit of Hg granules along the canalicular margin of hepatocytes.

CHAPTER 7
ELECTRORETINOGRAMS AND VISUAL EVOKED POTENTIALS

Introduction

Electroretinograms, which evaluate retinal function, and visual evoked potentials, which test cortical responses to visual stimuli, can provide a useful combination of tests for early visual dysfunction. Methylmercury can cause visual disturbances as an early sign of toxicity, as well as other CNS signs. Visual effects of MeHg in birds have not been well studied. The purpose of this portion of the project was to evaluate whether or not the visual system of cormorants exhibits early functional changes due to MeHg exposure, as measured by ERGs and VEPs. Funduscopic exams and intraocular pressure (IOP) measurements, as well as the ocular component of the neurologic exams (Chapter 8) were evaluated to further complement the clinical assessment of the visual system.

Methods

Electrophysiological testing was conducted at the Department of Ophthalmology Clinical ERG Laboratory, University of Florida, College of Veterinary Medicine. Each of the 22 cormorants underwent a set of three electrophysiological sessions, which included ERGs performed under scotopic and photopic conditions and followed by VEPs recorded under photopic conditions. The set of tests for each bird consisted of responses at a

predosing time, at 4 weeks, and at 8 weeks of dosing. Each testing session required 2 to 3 hours to complete. As a result only 2 birds could effectively be tested each day. A protocol was designed that staggered the dosing and electrophysiology testing and, in effect, allowed each bird to have responses recorded at exactly 0, 4, and 8 weeks. Time 0 corresponded to approximately 8-9 weeks of age. The oldest birds were approximately 2 weeks older than the youngest birds in the group. Based on plumage, body weights, and behavior, older birds were selected for testing first. Testing proceeded from the oldest to the youngest in order to more closely synchronize ages at time 0, the time of the first ERGs and VEPs and the beginning of mercury dosing.

Anesthesia and Mydriasis

ERGs and VEPs were performed under general anesthesia with isofluorane, and birds were placed in left lateral recumbency. Anesthesia records were maintained and included monitoring heart rate, respiration, and temperature, as well as oxygen and anesthetic flow rates. Birds were kept on a heating pad due to their tendency to lose body heat. Although most birds would breathe on their own, manual ventilation was generally used because a more even level of anesthesia was maintained and lower concentrations of anesthetic were needed to maintain an adequate level.

Adequate mydriasis was achieved with a combination of atropine, phenylephrine, and vecuronium bromide (Norcuron®, Organon Incorporated, West Orange, NJ). Vecuronium bromide is a curariform, nondepolarizing neuromuscular blocking agent. The inconsistant nature of pupil dilation in the juvenile cormorants, when using vecuronium as the sole mydriatic, necessitated a separate study (Loerzel et al., 1999) to determine the

optimal combination of drugs that resulted in the most stable dilation. The right eye was treated initially with three drops of atropine at 15-minute intervals, with a drop of phenylephrine approximately 2 minutes after the first and second doses of atropine. Following this "pretreatment" 3 to 4 drops of 4 mg/ml vecuronium bromide were administered. The status of the pupil diameter was monitored and recorded during testing and changes or irregularities were noted.

Intraocular Pressures and Ocular Exams

Immediately following anesthetic induction and intubation, each subject had IOPs measured in both eyes using a tonometer (Tono-Pen Model II, Oculab, Glendale, CA). Intraocular pressures were recorded at each electrophysiology session (0, 4, and 8 weeks) and evaluated for differences between treatment, across time, and between eyes (since the right eye was treated with mydriatics and the left was not). Ocular exams using direct ophthalmoscopy were also performed on both eyes at this time.

ERGs

ERGs were conducted on the right eye using Retinographics® (Norwalk, CT) software integrated, via the RetinoGraphics BPM-100 system, with a Grass PS22 photostimulator (Grass Medical Instruments, Quincy, MA). The light source was a Grass xenon strobe light modified with a 1/8" white dome-shaped plexiglass structure which acted as a diffuser. The light source was placed 1 cm from the eye. An attachment on the strobe light allowed for insertion of neutral density filters to control the light intensity. Responses were recorded using three electrodes including a positive corneal contact lens electrode (ERG-jet, Electrode Store, Yukoma, CA), a reference electrode at the lateral

canthus, and ground electrode at the midline caudal aspect of the skull. A 2.5% hydroxypropyl methylcellulose solution (Goniosoft, Ocusoft Inc., Richmond, TX) was used when applying the contact electrode. Platinum subdermal needle electrodes were used for the reference and ground connections. The eyelids were kept open with a custom-made plastic coated wire speculum adapted specifically to these birds. The third eyelid was also prevented from rising by the speculum as well as by the placement of the corneal contact lens electrode. The status of the nictitating membrane was monitored during testing to prevent any effects on the measured responses.

Preliminary studies determined that dark adaptation occurred within 5-10 minutes and that no increase in sensitivity occurred even when dark adapted for more than one hour. Based on these results, a conservative time frame of 20 minutes was selected for dark adaptation. Preliminary work also ascertained that a 1-minute inter-test time did not degrade or otherwise affect subsequent responses.

Profiles of ERGs were recorded after 20 minutes of dark adaptation and consisted of single flashes (10 μs duration) with 1-minute inter-test times. The response was recorded for 500 ms duration following the stimulus. Testing began at a no response level of light intensity, usually -3.2 relative log units, and increased in approximately 0.2 log unit increments (according to the neutral density filter values) to a maximal luminance stimulus of 8.05 cd·s /m^2 (total 17 tests per profile). Combinations of neutral density filters (Kodak-Wratten gelatin) were used to control the light intensity. The precise light intensity produced by the light source and each filter combination was documented (Figure 7.1). Background light levels in the darkroom were 0.001 - 0.002 cd/m^2 (detection limit of

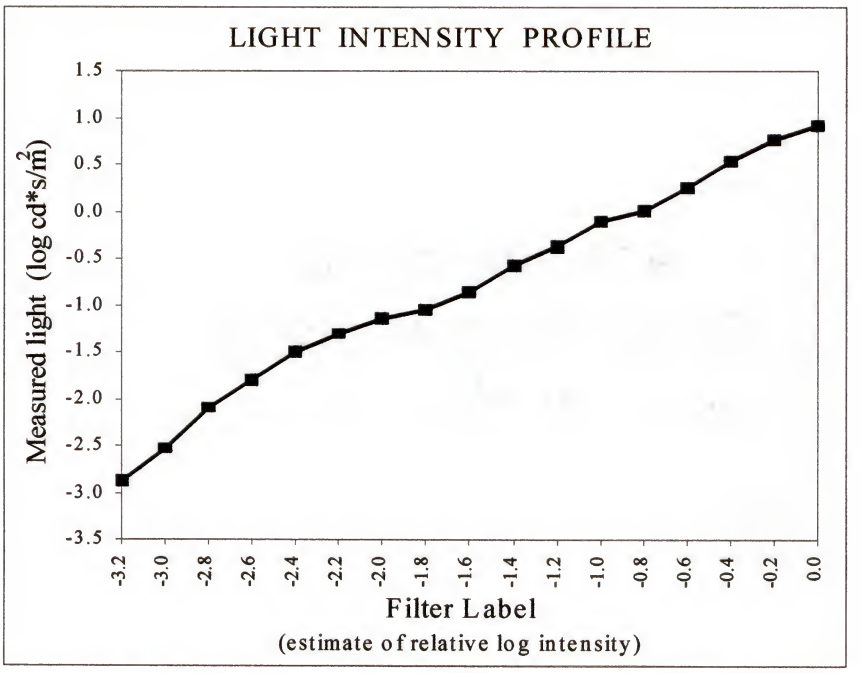


Figure 7.1 : Light intensity profile used in ERG and VEP protocols. Filters were approximations of the log of the relative intensities and actual light levels were verified using a calibrated photometer.

the photometer). The calibration of the light source was checked throughout the study to verify consistent light stimuli. Calibration and other light measurements were recorded using a System S350 Photometer (Graseby, Optronics, Orlando, FL). Following the dark-adapted testing, birds were light adapted in ambient light of 20-25 cd/m^2, measured at the eye position, for 10 minutes. The entire profile, as described for the dark-adapted testing, was repeated under light-adapted conditions.

VEPs

VEPs were conducted under light-adapted conditions using the maximal light stimulus. Subdermal electrodes were used and a set of six VEPs was performed for each bird. The four electrode placements which were selected included the following pairs of active (+) and reference (-) electrodes, respectively: OT1-Ch, OT2-Ch, SC-Ch, and SC-Fr

(Figure 7.2). The abbreviations OT, Ch, SC, and Fr stand for optic tectum, chin, sagittal crest, and frontal areas, respectively. A ground electrode (Gr) was place at the midline caudal aspect of the skull. For the first placement, OT1-Ch, responses to three light intensities (-1.05, -0.10, and 0.91 log (cd·sec/m^2)) were recorded for comparison of responses at low-, medium-, and high-level light stimuli. Responses at the other three electrode placements – OT2-Ch, SC-Ch, and SC-Fr – were recorded only at the highest intensity, 0.91 log(cd·sec/m^2). The OT1 and OT2 electrode positions were located just anterior to the ear opening, contralateral and ipsilateral to the eye receiving the stimulus, respectively. The OT placements corresponded to an area overlying the location of the optic tectum, and SC was overlying the hyperstriatal area of the brain. Each VEP response was a signal average of 32 flashes at 2-second intervals recorded for 500 mses.

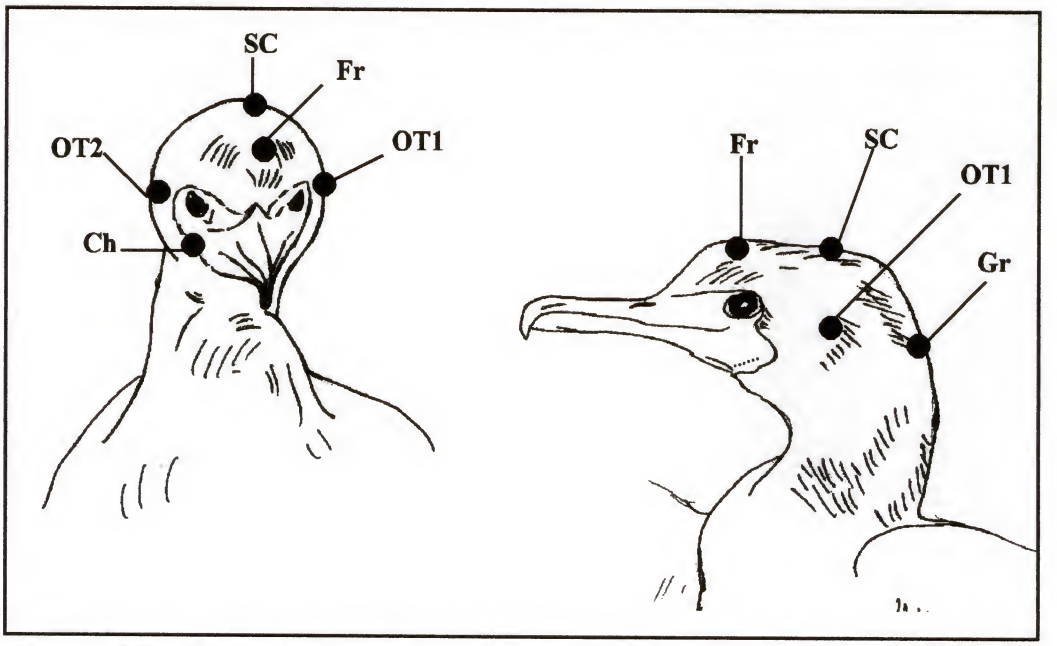


Figure 7.2 : VEP electrode placement. Four pairs were used and included the following active (+) and reference (-) electrodes, respectively: OT1-Ch, OT2-Ch, SC-Ch, and SC-Fr. Gr was the ground electrode. VEPs at OT1-Ch were performed at three light intensities, -1.05, -0.10, and 0.91 log(cd·sec/m^2).

Naka-Rushton Equation and Statistics

The a- and b-waveform parameters were measured as a function of light intensity. The ERG a-wave amplitude was measured from the baseline to the trough of the a-wave, and the b-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave. When no a-wave was discernible, the b-wave was measured from baseline to its apex. Latencies were measured from the stimulus onset to the a- or b-wave peaks. Descriptive statistics were summarized and an intensity response profile plotted for each of the ERG parameters under both photopic and scotopic light conditions. ERG a- and b-wave latency and amplitude responses from three light-intensity levels (0.54, -0.10, and -1.31 log cd·s/m^2) were analyzed for treatment-related differences using repeated measures ANOVA and Tukey-Kramer multiple pairwise comparisons. VEP P1 and N1 peak amplitudes and latencies were similarly evaluated. Differences were reported as significant for a $p \leq 0.05$ level, unless otherwise stated.

Scotopic and photopic b-wave amplitude intensity response profiles were further characterized by the Naka-Rushton equation,

$$R(I) = \frac{R_{max} \times I^n}{I^n + K^n} .$$

The Naka-Rushton equation is a nonlinear mixed-effects regression model where I is the stimulus intensity used to record the b-wave amplitude R(I). R_{max} is the theoretical maximum response amplitude, k is the stimulus intensity at which semi-saturation occurs, and n is indicative of the slope. This mixed-effects nonlinear regression (SAS, PROC MIXNLIN, Vonesh and Carter, 1992) was used to obtain R_{max}, k, and n estimates, with

95% confidence intervals, for a baseline assessment of the model fit using all 22 subjects prior to dosing. Following treatment with methylmercury, R_{max}, k, and n were similarly obtained for each treatment group at each of three time points (0, 4, and 8 weeks), then analyzed using repeated measures ANOVA and multiple pairwise comparisons (SAS, 6.1).

The analytical procedure for the b-wave amplitude response profile consists of a two-component model. First, it assumes the existence of an underlying population profile which can be modeled as a nonlinear mathematical function of independent variables. The Naka-Rushton equation would correspond to this level of the analysis, with luminance as the independent variable. The second component of the model assumes that the individual subject profiles will vary from the population profile in a manner that can be modeled as a function of the covariance structure which characterizes the variability of the Naka-Rushton parameters. The average model R^2 and the conditional model R^2 were also calculated. These numbers vary between 0 and 1, are somewhat analogous to the R^2 of linear regression, and represent goodness-of-fit indices for the population profile (average model R^2) and individual profile (conditional model R^2) components of the model.

Due to slight variability in pupil diameters, an additional analysis was conducted. Light intensity measurements were converted to trolands by multiplying luminance by the pupil area (Le Grand, 1957). The light source was positioned $\leq$ 1 cm from the cornea such that most of the retina would be illuminated regardless of small differences in pupil size. However, the aperture size was potentially a confounding variable, and the statistical analysis with trolands provided information regarding the variability of ERG responses due to differences in pupil size.

Results

Intraocular Pressures and Ocular Exams

The results of IOP measurements are summarized in Table 7.1. There were no treatment-related differences in IOPs between groups and no time-related changes within any group, for either eye. Although most average IOPs for the right eye were larger, the only statistically significant difference was in group C between right and left eyes at time 0 ($p=0.034$). Group C right eye IOPs at weeks 4 and 8 were also significantly larger than the left eye at baseline.

Table 7.1 : Intraocular pressures (mm Hg) for left (OS) and right (OD) eyes, at three time points, 0, 4, and 8 weeks. A = control, B= 3.5, and C=0.5 mg MeHg/kg diet daily. The right eye was treated with mydriatics prior to IOP measurement.

Tx		OS 0	OS 4	OS 8	OD 0	OD 4	OD 8
A	Avg.	**13.0**	**10.3**	**11.1**	**16.0**	**13.4**	**12.6**
	SD	3.5	2.6	3.0	3.3	2.1	1.8
	n	7	7	7	7	7	7
	Min.	7	6	7	12	10	9
	Max.	18	15	17	22	16	15
B	Avg.	**11.0**	**11.1**	**10.8**	**13.0**	**13.6**	**14.0**
	SD	4.8	2.7	1.6	1.7	2.8	1.4
	n	8	8	8	8	8	8
	Min.	5	8	8	9	10	13
	Max.	22	16	14	15	17	17
C	Avg.	**8.98***	**9.1**	**9.0**	**15.7†**	**13.4†**	**13.3†**
	SD	2.7	2.0	2.6	4.3	2.0	2.4
	n	7	7	7	7	7	7
	Min.	5	6	5	8	10	9
	Max.	14	12	14	23	16	17

*,† Numbers with different symbols are statistically different from each other at $p \leq 0.05$ level. No other comparisons were significant (i.e., unmarked numbers were not significantly different from any other averages).

No ocular abnormalities or abnormalities of the fundus were seen in any birds. The fundus appeared much like that described for cormorants by Woods (1917). It appeared mostly light blue to blue-grey with a fairly long, black pecten which appeared somewhat conical in shape, with the narrow end located over the optic disc. Several orange-red choroidal vessels could be seen parallel to, and on either side of the pecten. The fovea, in the nasal region, appeared almost refractile with thin pale lines crossing each other in the middle, with a light thin rim around it. One bird, O-17 from group B, had what appeared as a small white bleb (thought to be part of the optic disc) near the base of the pecten. This was noted from the very first exam and did not change with time (see Chapter 4 regarding other abnormalities in this bird).

Baseline ERG Data and Model Fitting

Typical photopic and scotopic ERG waveforms can be seen in Figure 7.3. Figures 7.4 and 7.5 depict baseline ERG parameters as intensity response profiles for scotopic and photopic adaptations. The lowest light level at which a clear ERG waveform was detectable in the majority of birds was at -2.10 log cd·s/m^2 for scotopic and -1.81 log intensity for photopic conditions. Photopic b-wave amplitudes were slightly smaller than scotopic amplitudes at low light intensities, but at the higher light levels, photopic b-waves were larger. Scotopic b-wave amplitude, at baseline, averaged about 225 μV, while the photopic average was about 340μV. Photopic b-wave latencies were also shorter than scotopic b-wave latencies. Scotopic a-wave latencies were about 20 msec at low light levels and gradually decreased to 10-11 msec at the maximal stimulus. Scotopic b-wave

latencies were about 38 msec at -2.10 log luminance and, after an initial mild decrease, increased to ~ 48 msec at 0.91 log luminance. Photopic a- and b-wave latencies had trends similar to the scotopic values. The a-wave times decreased slightly, with increased light intensity, from about 14 to 10 msec, and b-wave times remained fairly consistent at about 27 to 32 msec.

Predicted b-wave response profiles, with 95% confidence intervals, for baseline scotopic and photopic conditions are shown in Figure 7.6. Table 7.2 summarizes the Naka-Rushton parameters at baseline for light intensity measured as log cd·s/m^2 compared to log trolands, which adjusts for pupil diameter. No appreciable difference between these two methods was found in the profile parameters. The k value was different for trolands than it was for luminance, but this was a reflection of the difference in the unit value.

The overall fit of the Nakka-Rushton model was very good, as indicated by the average and conditional model R^2 values. The average model concordance correlation, which represents an index of agreement between predicted and observed values, was also quite high, with values of 0.9207 and 0.9500 for scotopic and photopic luminance profiles, respectively. In general the photopic luminance profiles fit the model slightly better than the scotopic profiles, and the conditional model R^2 was larger than the average model R^2. These goodness-of-fit measures remained relatively unchanged throughout the study in all treatment groups.

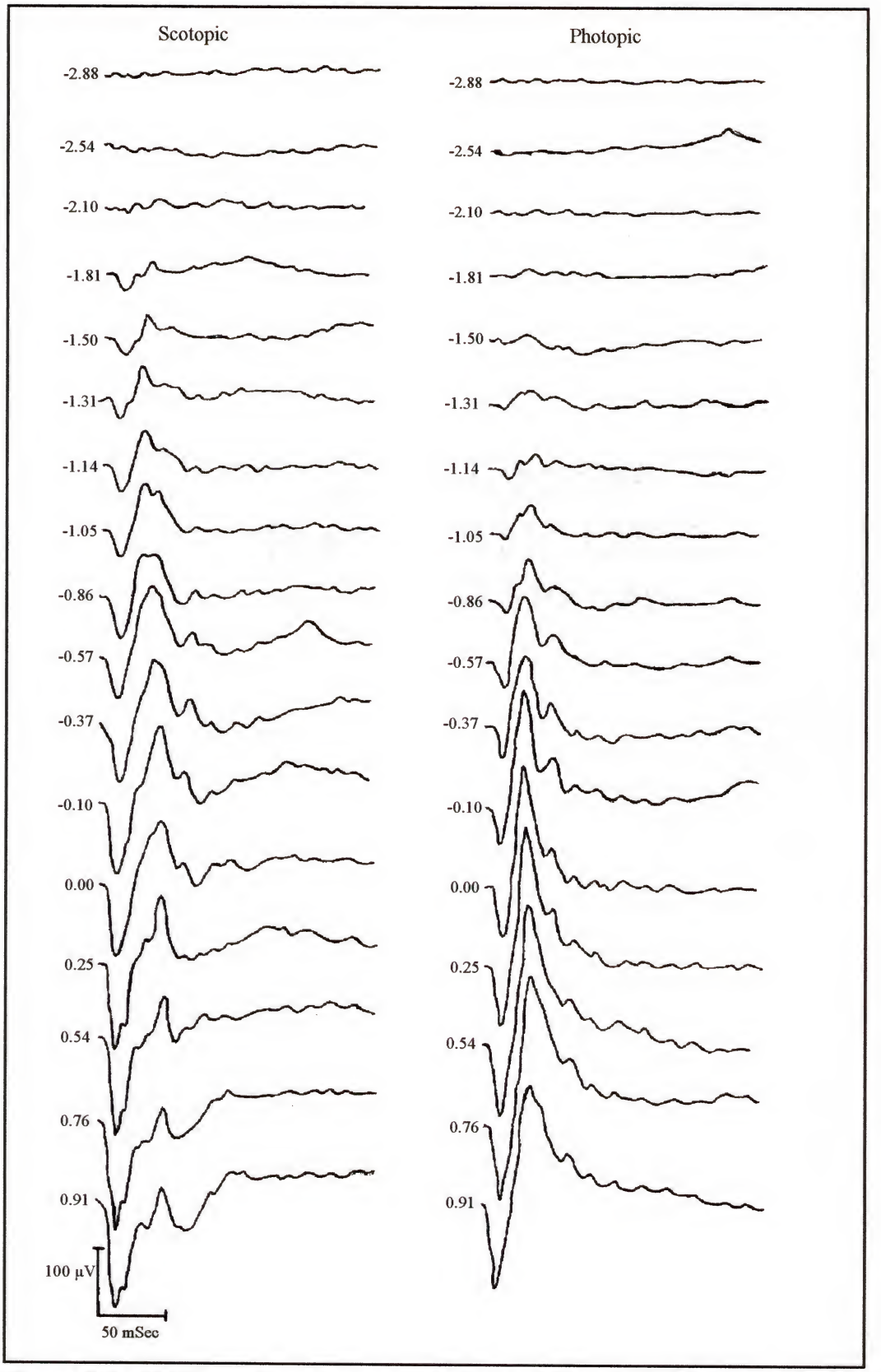


Figure 7.3 : ERG waveforms for scotopic (left) and photopic (right) conditions in a control bird at time 0. Log luminance ($cd \cdot s/m^2$) is indicated to the left of each wave.

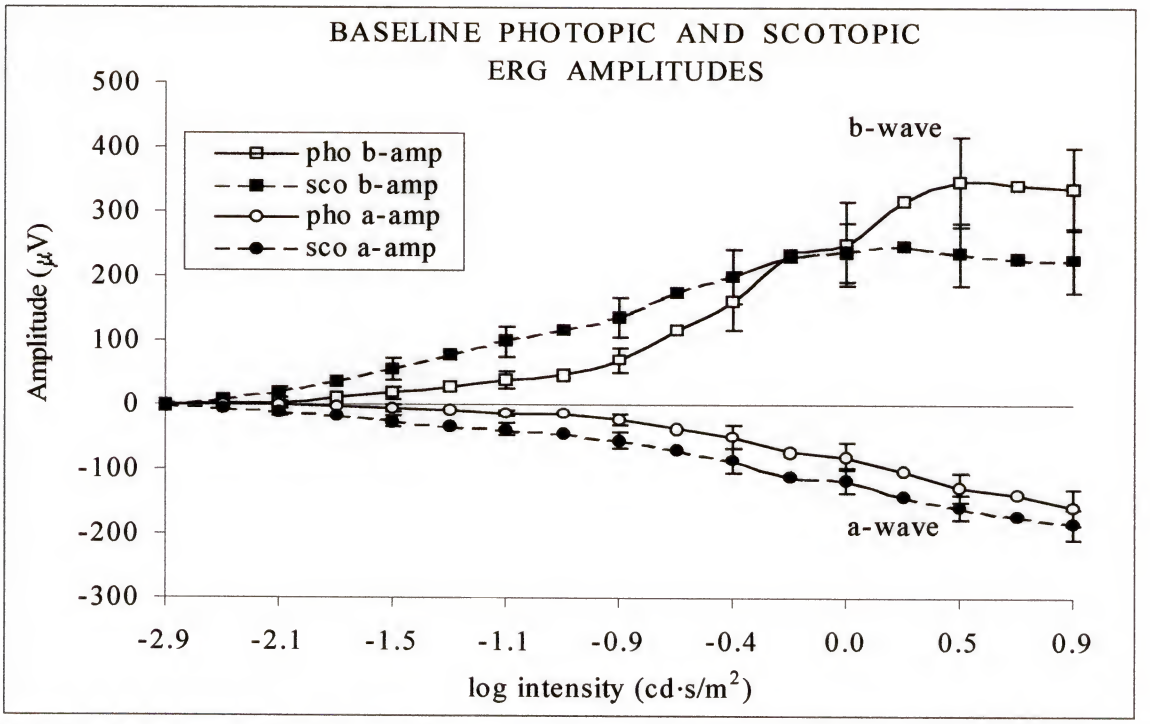


Figure 7.4 : Baseline ERG a- and b- wave amplitudes for 22 cormorants (approximately 8-9 weeks old). Open marks are photopic values, and filled marks with dashed lines are scotopic values. Bars represent ± 1SD. Maximum light intensity was 8.05 cd·s/m^2.

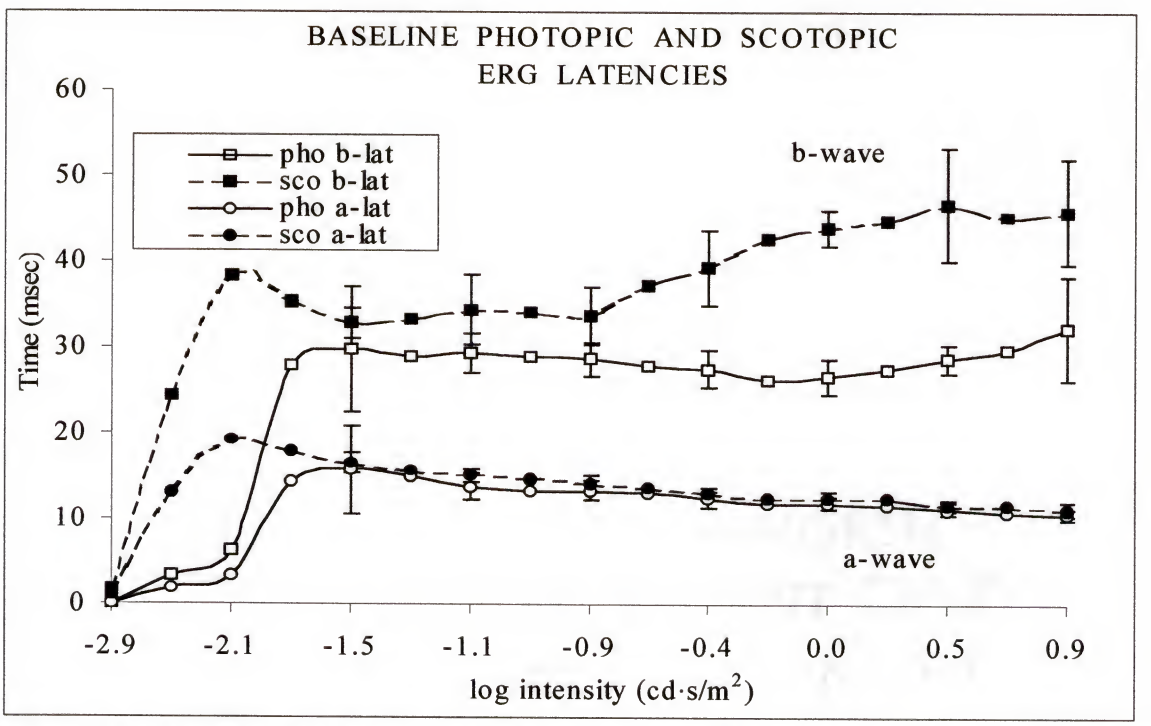


Figure 7.5 : Baseline ERG a- and b- wave latencies for 22 cormorants (approximately 8-9 weeks old). Open marks are photopic values, and filled marks with dashed lines are scotopic values. Bars represent ± 1SD. Maximum light intensity was 8.05 cd·s/m^2.

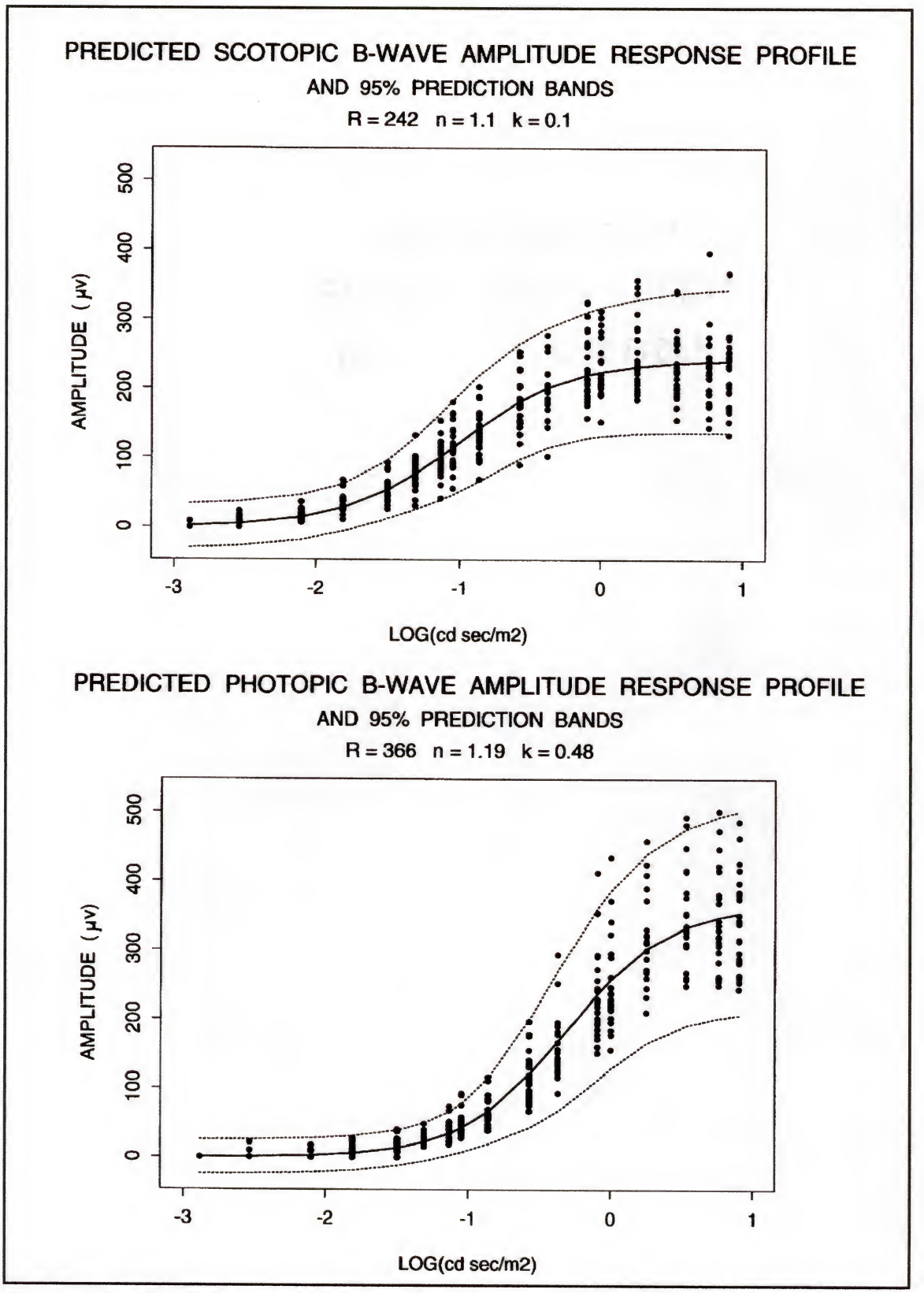


Figure 7.6 : Predicted scotopic (top) and photopic (bottom) b-wave amplitude response profiles for 22 birds at baseline time 0. R (R_{max}) is the theoretical maximum response amplitude, k is the semisaturation constant, and n represents the slope.

Table 7.2 : Baseline (22 birds) Naka-Rushton parameters and goodness-of-fit measures for photopic and scotopic conditions comparing results using log cd·s/m^2 and log trolands (adjustment for pupil size). Rmax is the theoretical maximum response amplitude (μV), k is the log intensity at which semisaturation occurs, and n represents the slope of the function.

Condition	Average Model R^2	Conditional Model R^2	n	Parameter	Estimate	SE	Lower 95% Limit	Upper 95% Limit
Photopic	0.9037	0.9778	371	Rmax	366	16	334	398
(cd·s/m^2)				n	1.19	0.03	1.13	1.25
				k	0.48	0.04	0.41	0.55
Photopic	0.8944	0.8742	371	Rmax	366	16	334	397
(troland)				n	1.18	0.03	1.11	1.25
				k	8.86	0.81	7.27	10.45
Scotopic	0.8515	0.9598	374	Rmax	242	11	220	263
(cd·s/m^2)				n	1.10	0.05	1.00	1.19
				k	0.10	0.01	0.08	0.12
Scotopic	0.8416	0.9683	374	Rmax	242	11	221	264
(troland)				n	1.09	0.05	1.00	1.19
				k	1.79	0.15	1.49	2.09

Treatment Outcome - ERGs

Naka-Rushton parameters

Results are reported for log luminence (cd·s/m^2) unless specified otherwise. No significant treatment effects, or treatment by time interaction effects, were observed (Figure 7.7). Significant time effects were observed for R_{max} under all adaptations, with

R_{max} at time 0 being significantly greater than at weeks 4 and 8 (no significant differences between week 4 and 8). Significant time main effects were also observed for k under all adaptations except photopic trolands, where the time main effect was marginally significant. Under scotopic adaptation, k was significantly greater at time 0 than at week 8, with k at week 4 having an intermediate magnitude which did not differ significantly from either week 0 or week 8. Under photopic adaptation, pairwise, contrasts of k among times were not significant. Under scotopic adaptation (trolands), k was significantly greater at week 0 than at week 4, with k at week 8 having an intermediate magnitude which did not differ significantly from either week 0 or 4. No treatment or time differences were seen in n.

B-wave amplitudes

No treatment effects were seen in photopic or scotopic b-wave amplitudes. A time main effect was apparent in both scotopic and photopic values. At time 0 scotopic b-wave amplitudes were significantly greater than those at week 8 at medium and high intensities, with those at week 4 having an intermediate in magnitude and not having differed significantly from either week 0 or 8. Photopic b-wave amplitude at week 0 was greater than that at week 4, at medium intensity, with the amplitude at week 8 having been intermediate in magnitude and not significantly different from either week 0 or 4 (Figure 7.8). Overall, at high intensity, b-wave amplitudes at week 0 were significantly greater than those at weeks 4 and 8.

B-wave latencies

There were no treatment effects seen in scotopic or photopic b-wave latency measurements. Only significant time and intensity (mentioned previously) effects were

seen. There was a time effect for photopic b-wave latency, with the b-wave latency measurements at week 8 having been significantly greater than those at week 4, while the b-wave latencies at week 0 were intermediate in magnitude, not having differed significantly from values at either week 4 or 8.

A-wave amplitudes

There were no treatment effects seen in the measurements of either scotopic or photopic a-wave amplitudes. A time effect in photopic a-wave amplitudes, at medium intensity, was apparent with those at week 0 having been significantly greater than those at week 4 (Figure 7.8). The week 8 high-intensity a-wave amplitudes were intermediate in magnitude and not significantly different from either week 0 or week 4 values. At high intensity the a-wave amplitudes at week 0 were significantly greater than those at week 4 and 8.

A-wave latencies

No meaningful treatment effect was seen in either the scotopic or photopic a-wave latency measurements. At low intensity, the photopic a-wave latency for treatment A was significantly greater than that in treatment C ($p=0.0116$), but this difference was apparent at each observation time, including week 0 (Figure 7.8). The treatment by time interaction for the low intensity a-wave latencies was not significant ($p=0.330$), which means that within each treatment group there was no evidence to indicate that the latencies changed from the baseline values. Thus, although a significant difference was detected between treatment groups for photopic a-wave latency, little importance was attributed to this finding with respect to MeHg toxicity. At low intensity there was also a time effect in

photopic a-wave latencies, across all treatments, with values at week 0 having been significantly greater than those at week 4, with a-wave latencies at week 8 having been intermediate in magnitude and not significantly different from values at week 0 or 4.

Treatment Outcome - VEPs

A typical set of cormorant VEPs is shown in Figure 7.9. Amplitude and latency profiles for the three treatment groups across time are depicted in Figures 7.10 - 7.13. The main peaks which were seen consistently were the first positive (P1) and negative (N1) peaks. Additional positive and negative peaks were seen in responses from several electrode placements, but these were not found consistently. Only P1 and N1 were evaluated for differences.

VEP amplitudes

When comparing intensities (log -1.05, -0.10, and 0.91) at the OT1-Ch electrode placement, there were significant treatment-related differences for P1, but not for N1 amplitudes (repeated measures ANOVA $p=0.0515$ for a treatment * peak interaction, but only marginally significant $p=0.0684$ for treatment effect alone). The pairwise Tukey-Kramer comparisons showed that at medium light-intensity (-0.10 log), at OT1-Ch, the P1 amplitudes in treatment B on weeks 4 ($p=0.0016$) and 8 ($p=0.0395$) were significantly lower than that in controls (Figure 7.10). At high intensity, at OT1-Ch, the treatment B P1 amplitude was marginally lower ($p=0.0654$) than in controls on week 8. Group B P1 amplitude was also significantly lower than that in group C at 4 weeks for the OT1-Ch electrode placement at low and medium light intensities. The N1 amplitude at OT1-Ch, at high intensity also had a decreasing trend during weeks 4 and 8 in group B but this was

not statistically significant (Figure 7.9). There were no significant time-related changes within any treatment at any intensity for the OT1-Ch electrode placement.

When comparing different electrode placements, there were no differences in amplitudes among the treatments at any time. There was, however, a subtle treatment by electrode interaction (ANOVA p=0.0023 for treatment*electrode placement). Within the control group no significant changes were detected among the three time points for any electrode placement, but within groups B and C there were time-related changes for certain placements. For the SC-Ch N1peak, the amplitude for group B had a progressively declining trend with the values at week 0 and week 4 having been larger than that at week 8 (Figure 7.9), but with no significant differences from other treatments. Group B, SC-Fr, P1 amplitude was smaller at time 0 than that at week 4. In group C the SC-Fr P1 baseline amplitude was lower than that at 4 and 8 weeks.

VEP latencies

There were no changes within groups over time, and no treatment-related changes in latency values, at any of the three light intensities, for the OT1-Ch electrode placement (Figures 7.12 -7.13). When comparing electrode placements there was a subtle treatment by electrode placement interaction (ANOVA p=0.0062 for treatment*electrode). The only apparent treatment-related difference was at SC-Ch with the N1 latency in group B shorter than that in controls at 8 weeks of dosing (p=0.0226, Tukey-Kramer). The N1 latency in group A (controls) had no changes across time, while in groups B and C the N1 latency of SC-Fr was shorter at week 4 compared to that at the baseline for those groups. There were no treatment-related differences in P1 latencies between groups, at any time point, when comparing electrode placements OT1-Ch, OT2-Ch, SC-Ch, and SC-Fr.

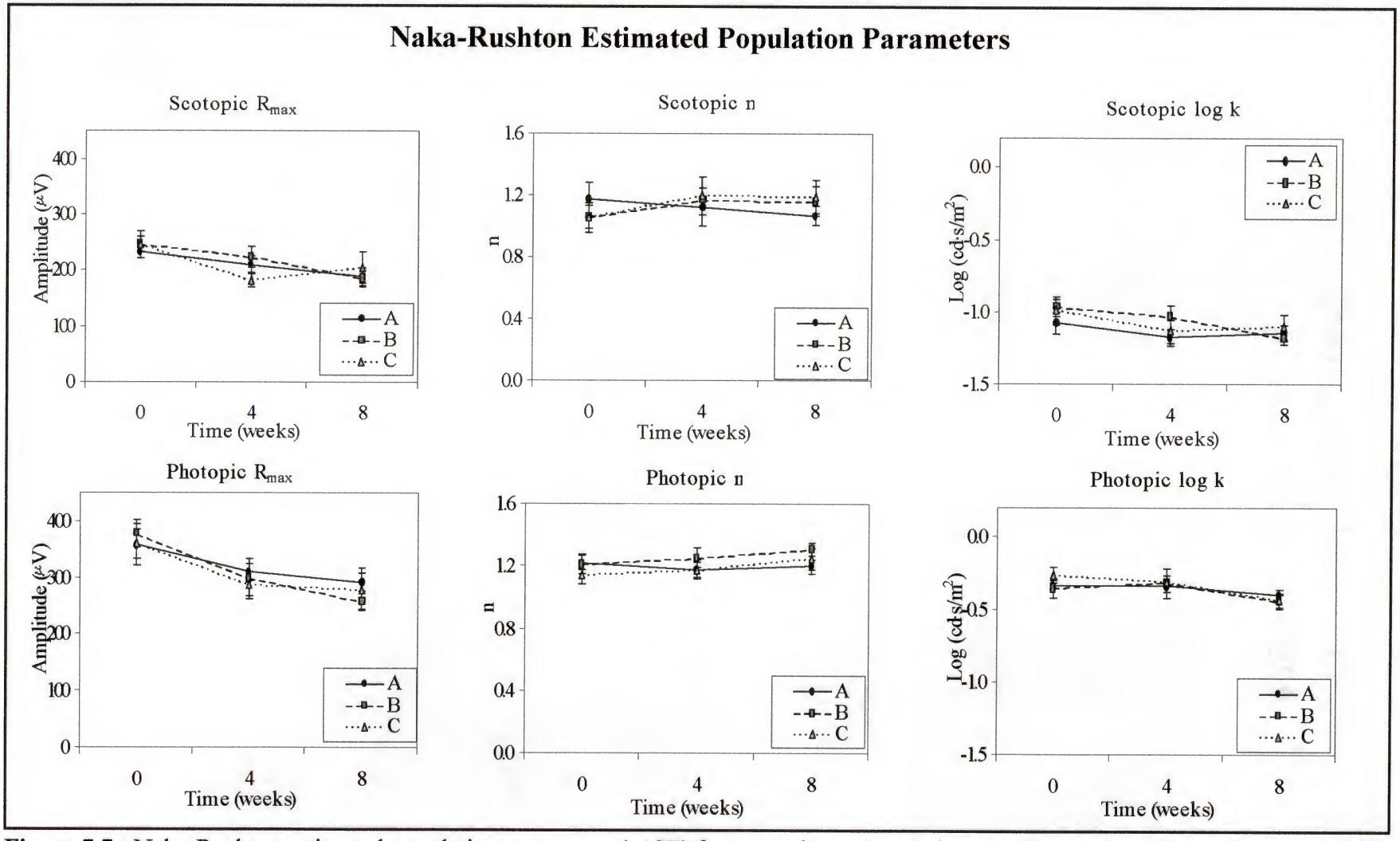


Figure 7.7 : Naka-Rushton estimated population parameters (±1SE) for scotopic (top) and photopic (bottom) conditions for three MeHg treatment groups at 0, 4, and 8 weeks of dosing. A - Control, B - 3.5, and C - 0.5 mg MeHg/kg fish/ day. No significant treatment-related differences were seen.

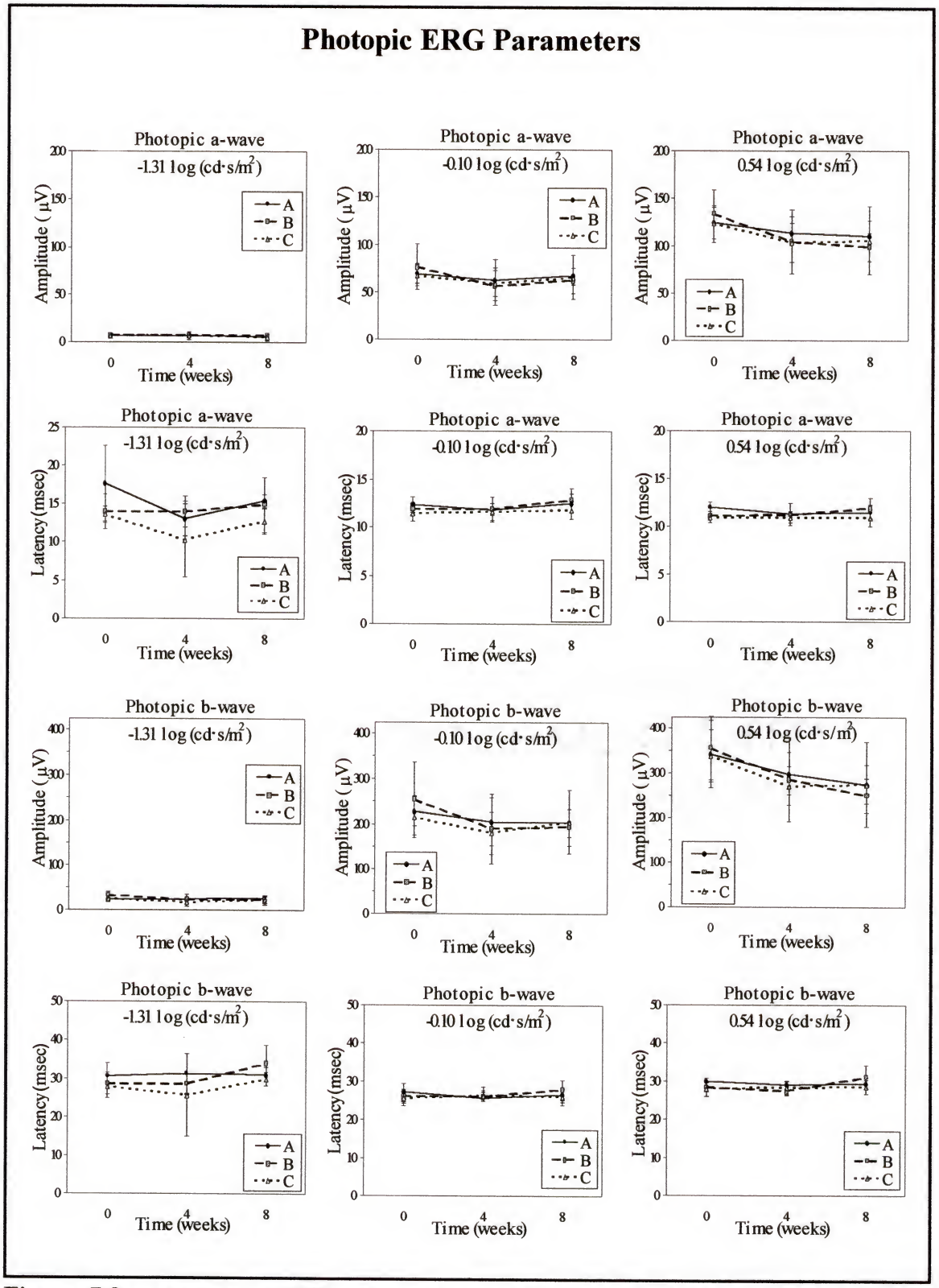


Figure 7.8 : Average (±1SD) photopic a- and b-wave amplitude and latency ERG parameters, at selected light intensities, for three MeHg treatments at 0, 4, and 8 weeks of dosing. No significant treatment-related changes were found in either photopic or scotopic a- or b-wave parameters. Scotopic data (not shown) had similar appearing graphs.

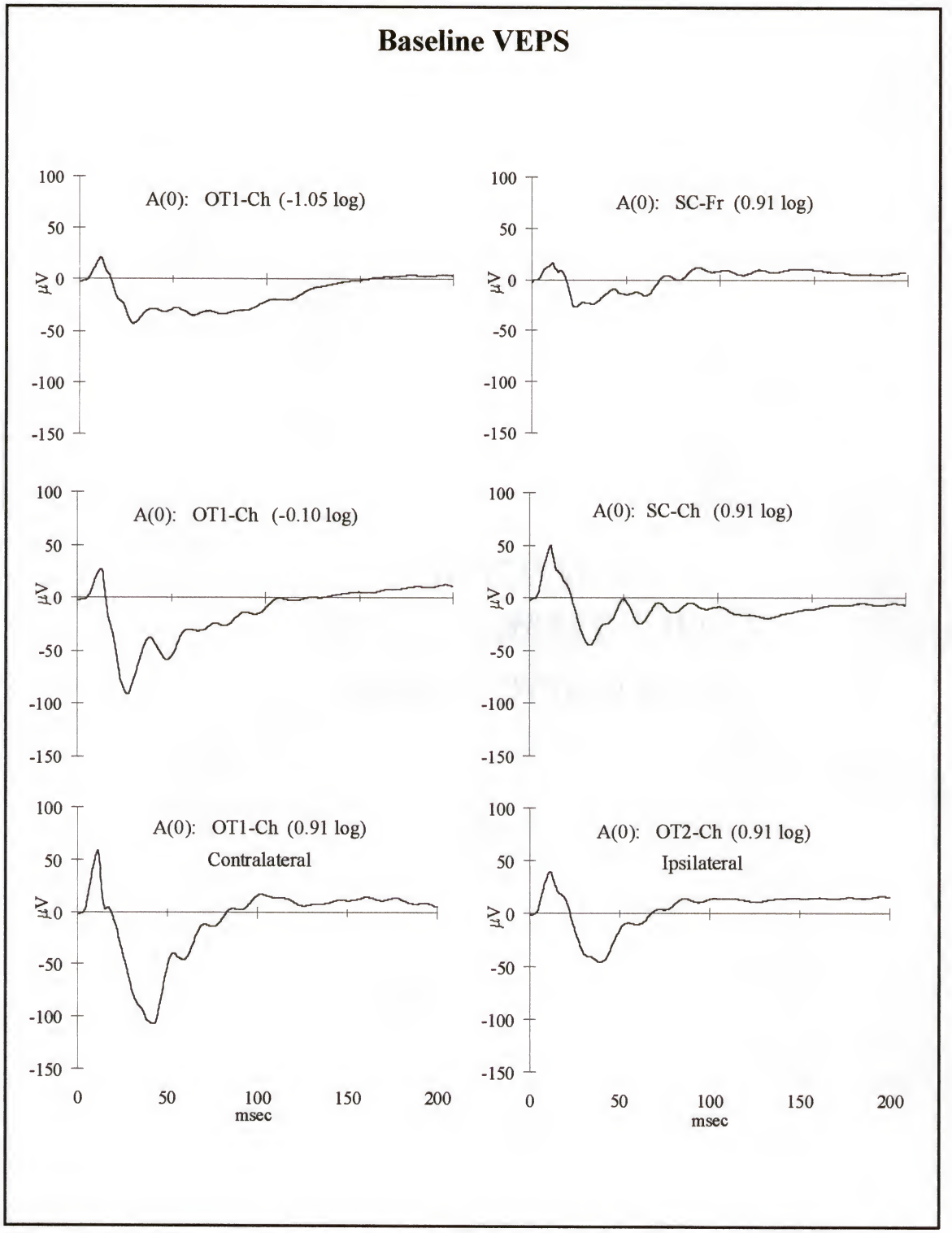


Figure 7.9 : VEPs at baseline time 0, averaged waveforms for 7 birds in control group A. See Figure 7.2 for description of electrode placement. The left column represents responses from one electrode set (OT1-Ch) at three light levels (-1.05, 0.10, and 0.91 log cd·s/m^2). The right column shows responses from three additional electrode placements.

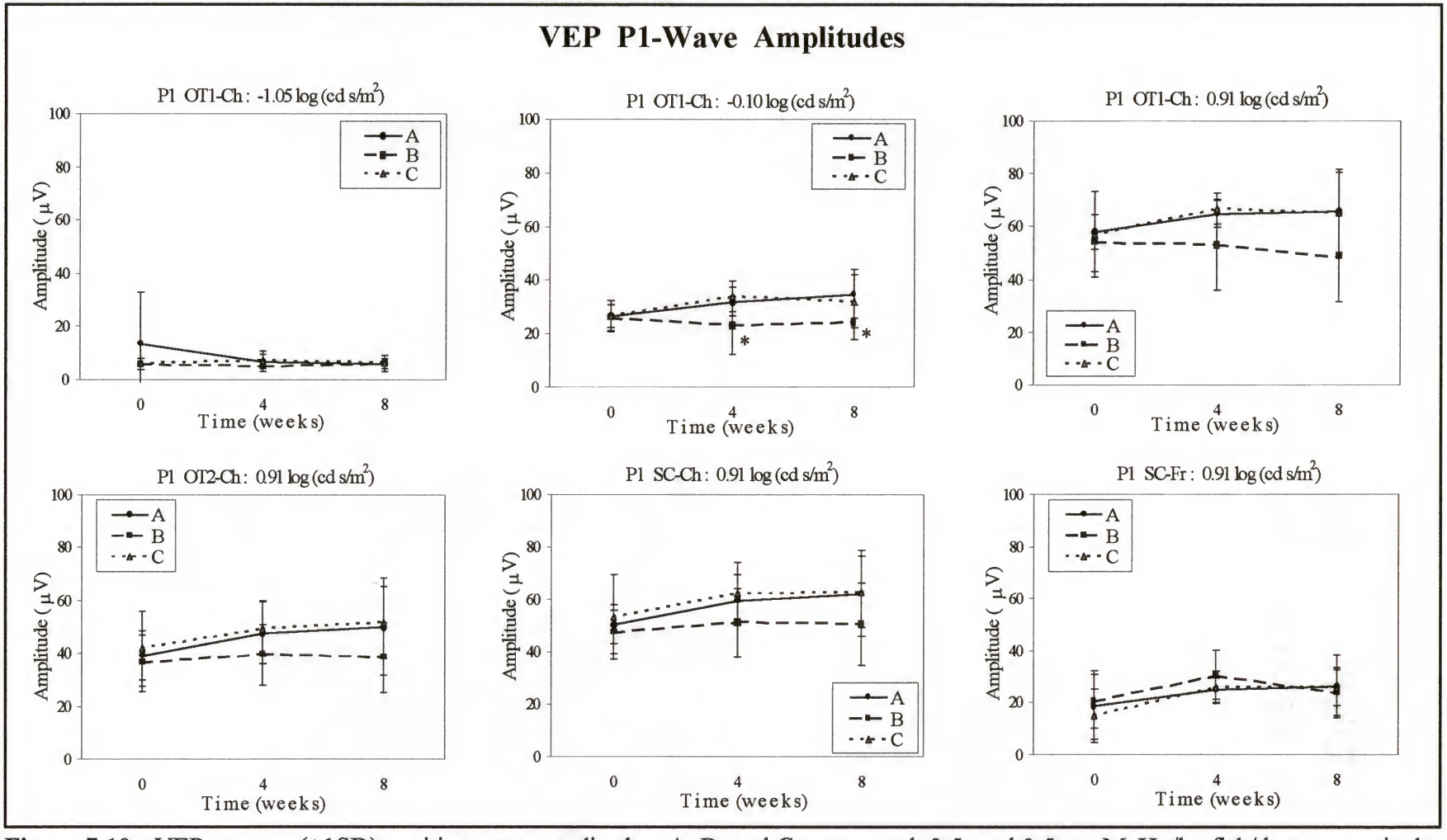


Figure 7.10 : VEP average (±1SD) positive wave amplitudes. A, B, and C are control, 3.5, and 0.5 mg MeHg/kg fish/day, respectively. Top three graphs represent responses at three different intensities (-1.05, -0.10, 0.91 log cd·s/m^2) for the same electrode placement (OT1-Ch), while the bottom three graphs are responses for three different electrode placements (OT2-Ch, SC-Ch, SC-Fr) all at the highest intensity. See Figure 7.2 for explanation of electrode placement. (*) significantly different than controls , $p \leq 0.05$.

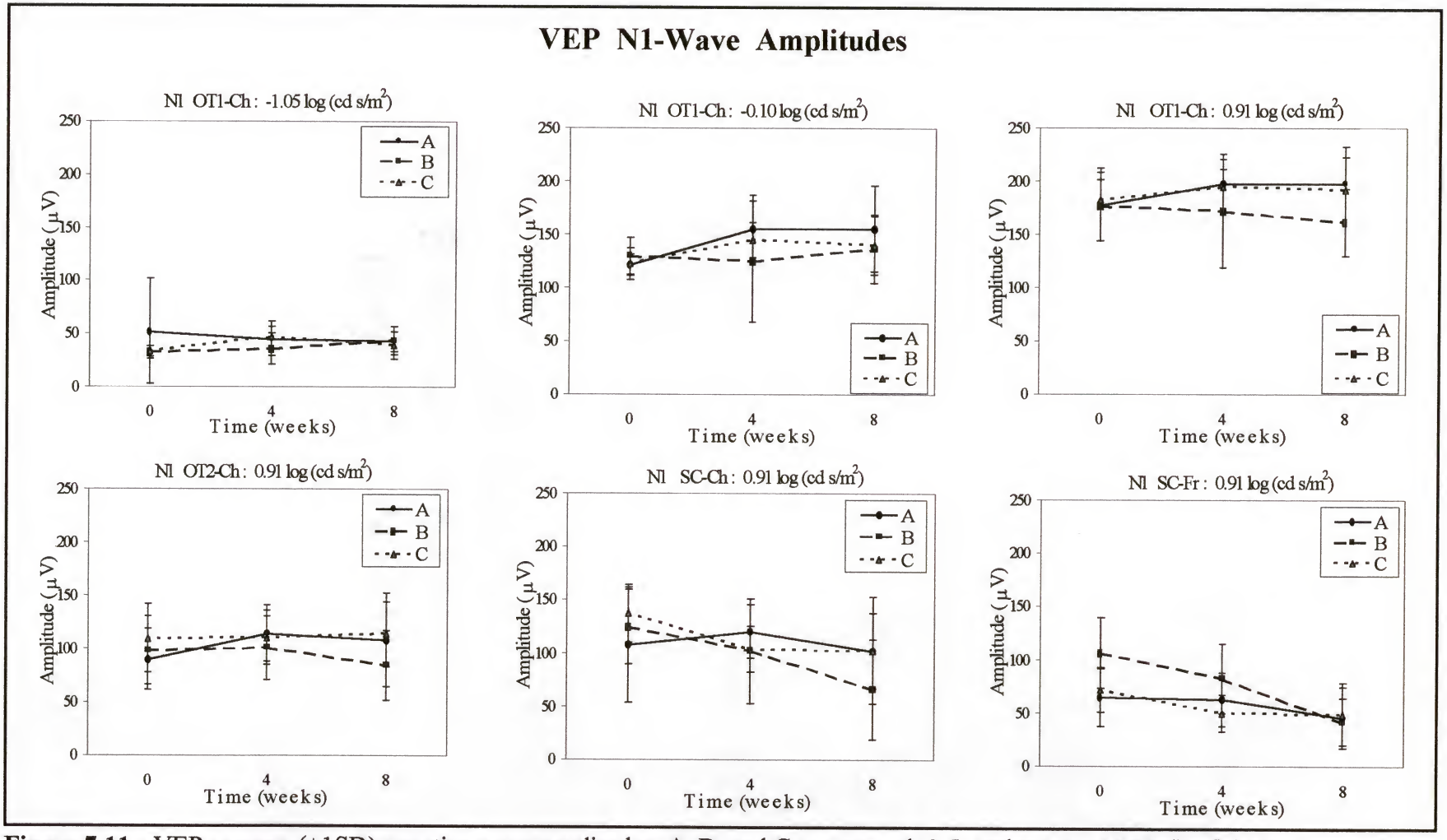


Figure 7.11 : VEP average (±1SD) negative wave amplitudes. A, B, and C are control, 3.5, and 0.5 mg MeHg/kg fish/day, respectively. Top three graphs represent responses at three different intensities (-1.05, -0.10, 0.91 log cd·s/m^2) for the same electrode placement (OT1-Ch), while the bottom three graphs are responses for three different electrode placements (OT2-Ch, SC-Ch, SC-Fr) all at the highest intensity. See Figure 7.2 for explanation of electrode placement.

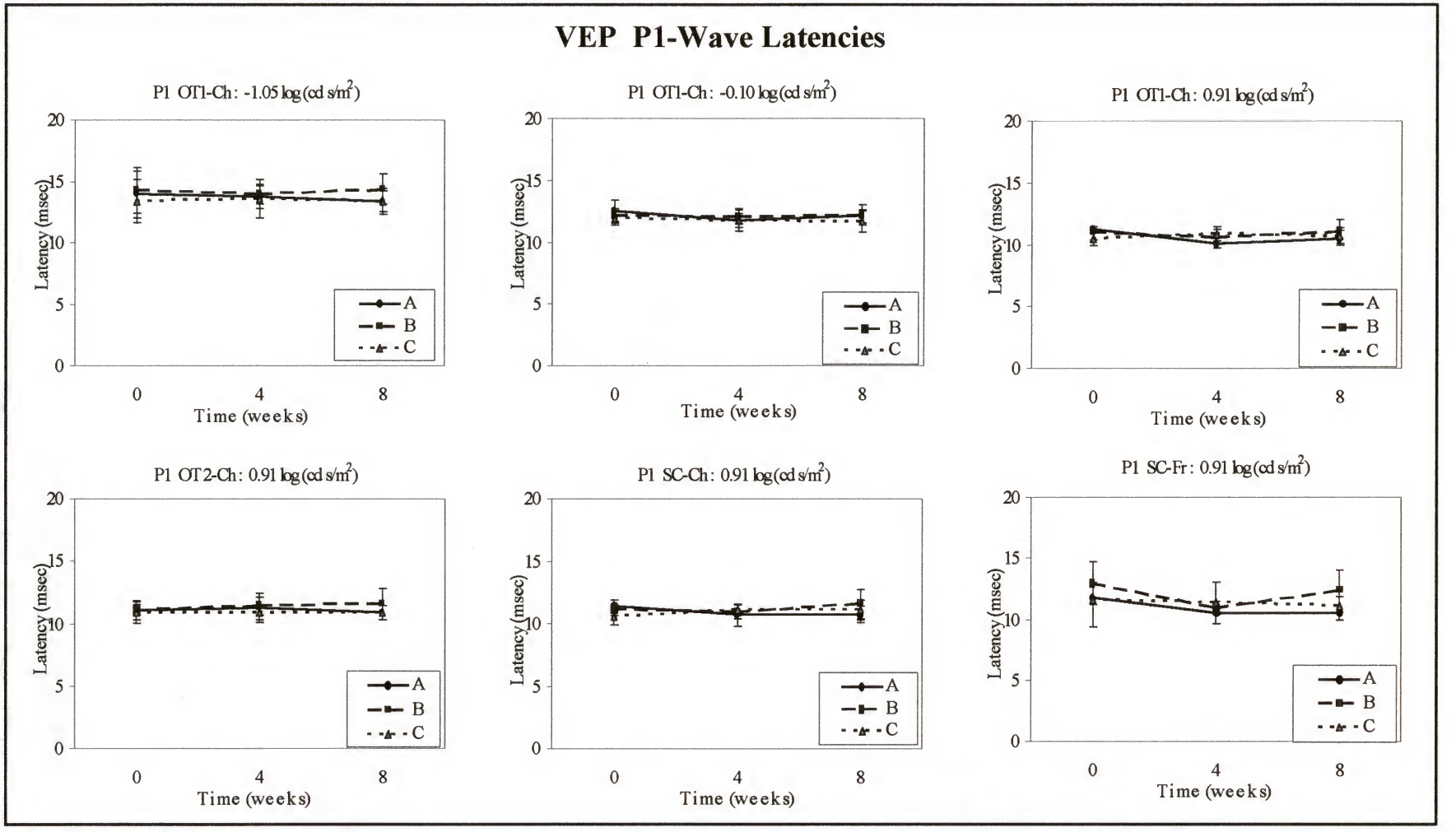


Figure 7.12 : VEP average (±1SD) positive wave latencies. A, B, and C are control, 3.5, and 0.5 mg MeHg/kg fish/day, respectively. The top three graphs represent responses at three different intensities (-1.05, -0.10, and 0.91 log cd·s/m^2) for the same electrode placement (OT1-Ch), while the bottom three graphs are responses for three additional electrode placements (OT2-Ch, SC-Ch, SC-Fr) all at the highest intensity. See Figure 7.2 for a description of the electrode placement.

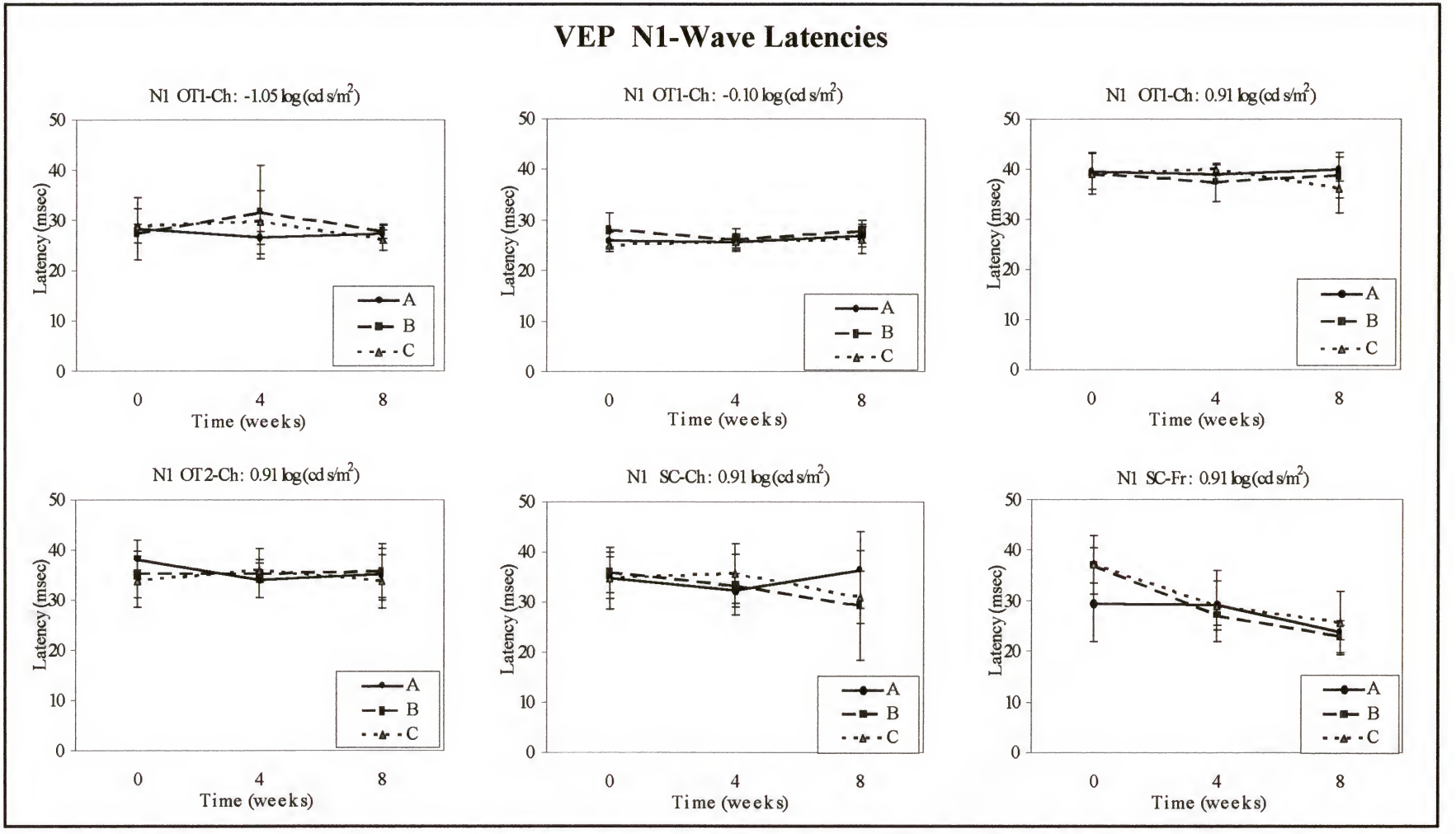


Figure 7.13 : VEP average (±1SD) negative wave latencies. A, B, and C are control, 3.5, and 0.5 mg MeHg/kg fish/day, respectively. The top three graphs represent responses at three different intensities (-1.05, -0.10, and 0.91 log cd·s/m^2) for the same electrode placement (OT1-Ch), while the bottom three graphs are responses for three additional electrode placements (OT2-Ch, SC-Ch, SC-Fr) all at the highest intensity. See Figure 7.2 for a description of the electrode placement.

Discussion

As in other species (Severns and Johnson, 1993; Gangadhar et al., 1989; Whittaker et al., 1996), the Naka-Rushton model describes the ERG profiles for cormorants quite well. The indices of goodness-of-fit remained consistently high throughout the study, which indicated the reproducibility of this type of characterization of the ERG profiles. The lack of changes in the fit of the model across time and treatment indicated that the level of variability did not change appreciably as a result of these factors.

A slight difference in the fit of the model was noticed between photopic and scotopic profiles with photopic responses having had slightly higher R^2 values than scotopic. This finding was likely a result of increased variability in scotopic b-wave amplitudes at high light intensities. At levels above 0.5 log ($cd \cdot s/m^2$) about half of the birds had b-wave amplitudes which were lower than the preceding responses. This type of response, with a dip in the b-wave amplitudes at higher intensities, has been described previously and often precedes a further rise in amplitude with increasing light stimulus (Severns and Johnson, 1993).

No treatment related changes could be detected in any of the ERG or Naka-Rushton parameters. In several instances, however, VEP P1 amplitudes were decreased in the high dose group compared to the controls, while no changes were seen in the low dose group. The significant treatment related effects were seen mainly in the OT1-Ch electrode placement, at medium and high light intensities. Although not statistically significant, a similar trend of decreasing P1 amplitudes could be seen in group B (high dose) compared to both A and C in several other instances. At OT2-Ch and SC-Ch the P1 amplitude was

affected in the high-dose group, and N1 amplitude at OT1-Ch (high light intensity) was also on a decreasing trend (Figured 7.10 -7.11). It is quite possible that these differences may have become more significant with prolonged dosing time.

Decreased VEP amplitudes is indicative of abnormal function in the post-retinal visual pathways. VEP latencies, compared to amplitudes, have generally been considered more reliable indicators of abnormalities, and can indicate lesions of the optic nerve, optic tracts or the visual cortex which affect conduction velocity (Seppäläinen, 1988). Visual evoked potential amplitudes may be altered with loss of conduction along axons or loss of active neurons in the visual cortex as well as from visual acuity changes.

Another interesting finding was that MeHg primarily affected the P1 amplitude of the VEP compared to a minimal effect on the N1amplitude. A study in rates indicated that different VEP peaks may have different generators and that recording a VEP overlying a visual cortex lesion results in effects on some peaks while other peaks may remain unchanged (Dyer et al., 1987). Holden (1968a) showed that early negative and positive waveforms could be generated from different areas of the superficial layers of the avian optic tectum. These waves were recorded at latencies of 2-3 msec, while in the cormorant the earliest positive waves were recorded much later (10-15 msec). No reports were available regarding the source of the individual VEP peaks when measured from the scalp.

The finding of decreased VEP amplitudes is consistent with studies of MeHg in other animals. VEPs in dogs exposed to methylmercury chloride exhibited decreased amplitudes (Mattsson et al., 1981). Specifically, the dogs had a distortion of the VEP after 1 week which stabilized until 5 -8 weeks, and deteriorated rapidly thereafter. No distortion

of the waveform was seen in the cormorants. In experimental methylmercury poisoning in rabbits, VEPs showed a reduction in amplitude (Iwata, 1980), and visual electro-corticograms in rats dosed with $HgCl_2$ had dose-related decreasing tendencies which only became significant after 12 weeks at a dose of 1.6 mg/kg (Dési et al., 1996).

In the OT1-Ch placement the active electrode is placed on an area corresponding to the optic tectum which plays a major role in central visual processing in birds. The optic nerve decussates completely in most birds, and thus an ipsilateral optic tectum impulse should, in theory, be negligible. However, in spite of the complete crossover of the optic nerves, there are components of the central avian visual pathways which provide bilateral input at the cortical level (Güntürkün et al., 1993, Engelage and Bischof, 1993). It has been shown that the ectostriatal region can receive visual input from both ipsilateral and contralateral stimuli, by way of fibers crossing over at the cortical level, and that VEPs from the ectostriatum receiving stimuli from the ipsilateral eye are about half the magnitude of the response from contralateral stimulus (Engelage and Bischof, 1993).

Visual evoked potential waveforms, as recorded in the present study, represent a composite of responses that cannot be easily separated. It is difficult to determine the exact origin of the various components of the VEP, especially when utilizing subdermal electrodes, because contributions to the responses include, or are affected by, many levels of the visual pathway including the ganglion cells and nerve fibers in the retina, the optic nerve, optic chiasm, optic tracts, optic tectum, etc. The majority of studies on cortical evoked potentials in birds have utilized more invasive methods using intracranial microelectrodes to directly measure potential within the cortex of the finch (Holden,

1968a; Holden, 1968b; Bagnoli et al., 1985; Engelage and Bischof, 1993), and no studies were found on the use of scalp electrodes for VEP measurements in birds. Waveforms obtained in this study differed from those obtained in the study of the ectostriatum by Engelage and Bischof (1993). In the zebra finch the intracranial VEP measurements resulted in waveforms with larger amplitudes and in some cases an N1 wave with longer latency time than that in the cormorants. Additionally, in the cormorants an initial positive peak (P1) was seen preceding the N1, while in the finch no short latency positive wave was seen. Data in other animals indicates that there is a substantial species variability in VEP responses with variations in the shape and times of the peaks (Creel et al., 1973). This type of species variability may also be present among birds.

Interestingly, in the current study the ipsilateral recording at OT2-Ch was about half the amplitude of the contralateral side, as was found in ectostriatal responses (Engelage and Bischof, 1993). Since VEPs have not typically been detected in the optic tectum ipsilaterally to the stimulus, but the ipsilateral ectostriatum has been shown to generated a VEP response, then a major contributing source of the ipsilateral response seen in the cormorants could have been from the ectostriatum. However, verification of the primary source of the generated potential, or the origin of the various components of a waveform which is measured form the scalp, would require further studies.

No data was available on ERGs or VEPs in cormorants specifically, but the ERG waveforms were similar in appearance to ERGs in other birds (Porciatti et al., 1989; Martin et al., 1975; Konishi and Homma, 1979; Nye, 1968). Cormorant ERGs differ from owls (Porciatti et al., 1989) in that the photopic b-wave amplitudes are larger than

scotopic. This is most likely indicative of the different proportion of rods versus cones in the retinas of these two birds. While no MeHg-related changes were seen in ERG or Naka-Rushton parameters, there was a time-related change in b-wave amplitudes across all treatments. Other reports in pigeons (Porciatti et al., 1985) and dogs (Gum et al., 1984) have indicated that age-related changes in b-wave amplitudes do occur. In pigeons ERG amplitudes increased to adult levels by 20 days of age, but no decrease was reported thereafter (Porciatti et al., 1985). In beagles the ERG amplitudes increase to adult levels by 8 - 9 weeks, after which they stabilize (Gum et al., 1984). In contrast to these studies, the cormorants exhibited decreasing amplitudes with age. This may have been a normal change, or some other factor, such as a nutritional deficiency, could potentially have affected all treatment groups. The latter was considered unlikely since adequate vitamin supplementation was provided to all birds throughout the study.

No MeHg-related changes in the fundus or intraocular pressures were found. No clinically apparent lesions were expected, although an early report on ocular manifestations of mercury poisoning indicated that minor changes, such as diminution of retinal blood vessels, may be present (Sabelaish and Hilmi, 1976). Intraocular pressures were also not expected to change, but in theory mercury could alter the ocular osmotic balance by inhibition of ion channels (Sirois and Atchison, 1996; Leonhardt et al., 1996), or altered divalent cation homeostasis (Denny and Atchison, 1996). Additionally, mercury was also localized in the ciliary epithelium (Chaper 6) in the high-dose group.

Although pupil dilation was slightly variable, analysis of the ERG data using trolands, which factors in pupil diameter, provided results that were almost the same as

when cd·s/m^2 were used as the stimulus intensity units. The proximity of the light source to the cornea probably provided close to maximal retinal illumination, and thus minor variations in pupil size did not appreciably affect the outcome. Both ERG and VEP waveforms were quite reproducible between test times.

In summary, the finding of altered VEP but not ERG parameters indicates that initial visual effects of MeHg are centrally mediated rather than peripherally in the retina. Changes in VEPs occurred before any overt signs of toxicity and may be a useful tool in evaluating early effects of MeHg exposure. Further study on the source of potentials recorded with scalp electrodes may help in localizing the most affected cortical areas.

CHAPTER 8
NEURO-BEHAVIORAL STUDY

Introduction

Careful assessment of the neurological and behavioral status of the cormorants was an integral part of this study. The intent of the electrophysiology tests was to evaluate whether they were affected in preclinical MeHg toxicity. In this context, systematic evaluation of neurological status was necessary in order to ascertain at what point clinical signs of toxicity first appear. The purpose of this portion of the experiment was to evaluate whether any birds developed neurological signs as a result of the MeHg exposure, and if so, which components of the neurologic exam were first affected and at what exposure time and level.

Behavioral observations were also performed as a supplement to the neurological exams. Neurologic exams tend to involve more handling (stress), which may inadvertently mask subtle changes in the status of the subjects. Standardized behavioral observations, with the observer removed from the immediate environment of the subject, provide an opportunity to detect more subtle alterations in the clinical status, such as activity level, social interactions, and attitude, of the birds. The behavioral observations conducted in this study represent the most common general behaviors that were observed in preliminary studies of these birds in captivity. Clearly, it would be risky to extrapolate the data

obtained in the captive state to behaviors in free-ranging birds, but learning more about the effects of methylmercury on the behavior of cormorants may help establish noninvasive methods of assessing mercury levels and its effects in wildlife. The purpose of the behavioral observations was to provide an added means of detecting subtle changes in the clinical status of the birds by first evaluating the range of common normal behaviors of captive cormorants, and then determine if exposure to methylmercury alters these behavior patterns. The primary goal was to conduct systematic behavioral observations and assess if any changes in the character of the behaviors and/or the time spent in particular behaviors occur as a result of exposure to a low and high dose of methylmercury for eight weeks.

Methods

The 22 cormorants included in the study were housed, fed, and treated as described in Chapter 3. The project remained double-blinded throughout. Standardization of techniques and categorization of responses was conduced for two to three weeks prior to dosing for both the neurological and behavioral observations.

Neurological Exams

Weekly neurological exams were performed on each individual. These exams consisted of an observational component prior to handling, then a systematic assessment of cranial nerves, reflexes, and postural reactions according to the format in Table 8.1, which is similar to the basic format for neurological exams in psitticines and other domesticated avian species (Clippinger et al., 1996) with a few exceptions. Adjustments were made due to species-specific differences in anatomy as well as typical responses to

Table 8.1 : Neurologic exam form. Exams were performed on each bird weekly.

Neurologic Examination

ID_____ Time_____
Date_____ Observer_____

General attitude, personality, and mental status: _____

_____

(Alert, depressed, fluffed feathers, sleepy, dull, uneasy, restless, nervous, anxious, aggressive, dizzy, disoriented, stupor, seizures, coma, etc.)

Vocalization:

Posture:

Head:

Wings:

Resting Posture: (L) (R)
Wing Spread (Drying Posture): (L) (R)

Legs:

Perching: (L) (R)
Standing: (L) (R)

Gait:

Flying:

Landing on perch:

Swimming:

Other: _____

_____

_____

-2 = None -1 = Diminished 0 = Normal 1 = Mild to Moderately Exaggerated
2 = Severely Exaggerated, clonus NE = Not Evaluated NA = Not Applicable

Wings and legs:

	Left	Right	Comments
Wing withdrawal			
Proprioception			
Hopping			
Leg Withdrawal			
Grip Strength			
Placing - Tactile			
Placing - Visual			
Wings Falling			
Landing on perch			

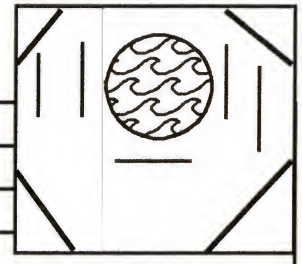
Eyes:

	Left	Right	Comments
Menace / Vision (II, VII)			
Following (II, III)			
Pupils resting (II, III) (symmetrical?)			
PLR - direct			
Palpebral (V, VII)			
Strabismus resting (II, IV, VI)			
Strabismus position (III, IV, VI, VII)			
Nystagmus (VIII)			

Table 8.2 : Examples of data collection forms for focal and scan sampling. The diagram in the upper right corner is a schematic of the pen layout, with pool and perches.

Date _____ ID _____
Time _____ Pen #_____
Observer_____

Focal Behavioral Observations



Time (seconds)

Behavior	0	30	60	90	120	150	180	210	240	270	300	Comments
Perching												
Standing												
Preening												
Sleeping												
Looking around												
Manipulating objects												
Interacting w/penmates												
Spreading wings												
Swimming												
Bathing												
Begging												
Other												

Date:
Time:

Scan Sample

ID	Perching	Standing	Preening	Sleeping	Looking Around	Manip. Objects	Vocali-zation	Interact w/penmat	Wing Spread	Swim	Bathing	Begging	Other
Y-01													
O-02													
R-03													
G-04													
B-05													

specific tests. For example, patellar and tibial reflexes could not be performed because the birds normally held their legs in a tightly flexed position when they were lifted from the ground, and thus no consistent response could be elicited.

Most components of the cranial nerve and spinal reflex tests were recorded as a ranked response from -2 (absent or severely depressed) to +2 (severely exaggerated), with 0 = "normal." Proprioception was ranked similarly but was based on the delay time of the response. The proprioceptive test was performed by curling the birds' toes under and placing the dorsum of the foot on the ground. A normal response for these birds was to correct the abnormal toe position almost immediately. A delay of 1-3 seconds was arbitrarily ranked as -0.5, 4-6 seconds as -1.0, 7-9 seconds as -1.5 and ≥10 seconds delay as -2. Any abnormalities in the observational aspect of the neurologic exam were described; otherwise responses were reported as "normal." Each neurologic exam was performed independently from prior exams, and sequential data was compiled only the end of the study.

Behavioral Observations

Based on preliminary observations prior to dosing, a series of behaviors was selected and defined for inclusion in the study. Focal and scan sample observations (Martin and Bateson, 1986) were conducted, in random order, 2-3 times weekly by one of two trained individuals. The observers recorded behaviors from behind a blind set about 15-20' from the pen being evaluated. Extenuating circumstances prevented the use of the blind on three occasions.

Focal samples consisted of observations of each individual in a pen for 5 minutes and recording behaviors at 30-second intervals (Table 8.2). Before and after each 5-minute focal sampling, a scan sample was taken of all birds in that particular pen, to get an overall view of what the entire group was doing. The scan sample involved recording the activities of all birds in the pen at that one time point (Table 8.2). Since it is difficult to avoid at least some anthropomorphism when describing animal behavior, use of quotation marks acknowledge this use in cases where an anthropomorphism may more easily and clearly convey an idea than a lengthy technical description.

The behaviors included in the study were selected based on the most commonly observed activities reported in the preliminary observations and were defined as follows:

1. Perching - located on a limb or the pool edge. Generally birds were in a standing position, bearing weight on both feet while perching. However, some individuals assumed a "sitting" or crouched position on their perch, which amounted to a bending of the legs so their keel rested on the perch. The "sitting" position was often observed when birds slept. Perching on one leg was also common.
2. Standing - located on the floor or the pen, either standing in one place or walking.
3. Preening - manipulation, cleaning, or maintaining the feathers with beak or feet, including scratching head with feet.
4. Sleeping - resting with eyes closed, most often with head folded across back with face tucked in feathers. This category also included birds which "sat" facing forward in a relaxed position with eyes closed.

5. Looking around - showing active interest in surroundings as indicated by turning head to face an object, focusing attention in a specific area, or fixing gaze on some individual or object.
6. Manipulating objects - picking objects up with beak or feet, tossing articles into air, or biting at objects, including astroturf, strings, twigs, or plastic liner.
7. Vocalizing - making any type of sound.
8. Interacting with penmates - actively engaging in an activity with another bird, such as biting at feet, wings, or beak. This included intertwining of body parts (necks), grooming each other, or "playing" with sticks, or other objects, together.
9. Spreading wings - perching or standing with wings either partially or fully opened, usually to dry wings, although this behavior was sometimes seen when birds were not wet.
10. Swimming - floating and paddling in a relaxed manner in the wading pool, sometimes included "ducking" head under water and looking around under water.
11. Bathing - swimming while vigorously ruffling feathers, splashing, and dipping head repeatedly under the surface of the water.
12. Begging - approaching a human with head elevated, neck extended, spreading and flapping wings excitedly, and vocalizing (DeLaRonde and Greichus, 1972).

Components of the neurological tests in which abnormal responses were noted were analyzed for differences among groups using the Kruskal-Wallis test followed by Wilcoxon Rank Sum test for pairwise comparison between groups. The Friedman Rank Sum test and the Wilcoxon Signed Rank test were used to evaluate for an overall time

effect and for change from baseline significance, respectively. Descriptive statistics were used to evaluate the observed behaviors, and statistical analysis was done on arc sine square root transformed data using repeated measures ANOVA and Tukey-Kramer multiple pairwise comparisons (PROC GLM, SAS 6.1). The behaviors seen in the control group were described and then compared and contrasted with the MeHg-treated groups.

Results

Neurological Exam

The proprioceptive test in the high-dose group was the only component of the neurological exam that was affected consistently as a result of MeHg treatment. In groups A and C birds were occasionally reported as having mild proprioceptive deficits but with no clear pattern (Table 8.3). One bird, O-12 in group C, had severe deficits in its right foot due to a joint injury (details in Chapter 4). In group B (high dose), however, significant deficits appeared in the majority of birds starting at week 5 of dosing. In most cases, deficits in this group were bilaterally symmetrical, although a few exceptions occurred.

All ocular components of the neurological were normal throughout testing. Likewise, there were no abnormalities noted in wing withdrawal or placing-visual tests at any time. For the remaining tests – including hopping, leg withdrawal, placing-tactile, grip strength, and wings-falling – scattered mild deficits were reported throughout the study. These irregularities had no clear pattern, involved all treatment groups, and no alteration in responses could be definitively attributed to MeHg dosing. There were a few individual cases in the tests mentioned above that had some pattern or suggestion of a pattern. In group B, two birds had bilaterally symmetrical, mildly delayed hopping responses (-0.5) in

weeks 7 and 8. Group C bird O-02 had depressed leg-withdrawal responses bilaterally on weeks 3, 5, 6, 7, and 8. Bird O-17 in group B had weakened grip strength on all but weeks 1 and 2, and abnormal wing responses, when dropped from a standardized height on the wings-falling test, during the entire study.

When evaluating how well birds landed after flying a short distance, three birds from group B were found to have poor-quality landings during weeks 7 and 8. One of those birds also had bad landings as early as week 5. Bird O-17 (B) also had poor landings for the entire study, and O-12 (C) had problems landing on weeks 2, 6, and 7. The results from these two birds were not weighted heavily in the landing evaluation since they had other physical problems (Chapter 4). In group C, one bird had abnormal landings in weeks 7 and 8, but no other birds in either the controls or group C had landing problems.

Behavioral Data

A summary of selected results for focal behavioral observations is presented in Table 8.4. The birds in the control group spent approximately 95% of their time perching initially, and the mercury-dosed birds had comparable values of 85% and 88 % for low and high doses respectively. Results from week to week were somewhat variable. On average, for all weeks combined, group A spent about 84% of the time perching, with similar overall averages for groups B (86%) and C (82 %). For week 8 group B spent less time perching (68%) that at any other time point for that group. Groups A and C also had relatively low perching rates of 78% and 75%, respectively, but these groups had similar values in prior weeks also. Scan sampling resulted in a similar pattern of time distribution among the various activities.

Table 8.3 : Conscious proprioception in control (A), low- (C), and high- (B) dose MeHg-treated cormorants. L = left, R = right. Values are ranks: 0 = normal, -0.5 = delay of 1-3 seconds, -1 = delay of 4-6 seconds, -1.5 = delay of 7-9 seconds, -2 = delay ≥10 seconds. Exams were performed at the end of each treatment week. Group averages and standard deviations (SD) are summarized below each set.

End of week:			0		1		2		3		4		5		6		7		8	
ID	Tx	Sex	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
G-19	A	F	0	0	0	0	0	0	0	0	-1	0	0	0	-1.5	0.5	-1	0	-1	-1
Y-06	A	F	0	0	-1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G-09	A	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G-14	A	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Y-01	A	M	0	0	0	0	0	0	0	0	0	0	0	0	-0.5	-0.5	0	0	0	0
Y-11	A	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-0.5	-0.5
Y-16	A	M	0	0	0	0	0	0	0	0	0	0	0	0	0	-1	0	-1	0	0
		Avg.	0.00	0.00	-0.14	-0.14	0.00	0.00	0.00	0.00	-0.14	0.00	0.00	0.00	-0.29	-0.14	-0.14	-0.14	-0.21	-0.21
		SD	0.00	0.00	0.35	0.35	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.52	0.44	0.35	0.35	0.36	0.36
B-05	B	F	0	0	0	0	0	0	0	-2	0	0	-1	0	0	-1	-1	-1	0	-1
B-10	B	F	0	0	0	0	0	0	0	0	0	0	-1	-1	-1	-1	-1.5	-1.5	0	-0.5
B-20	B	F	0	0	-1	-1	0	0	0	0	-1	0	0	0	-0.5	-0.5	-0.5	-0.5	-1	-1
B-25	B	F	0	0	0	0	0	0	0	0	0	0	-1	-1	-0.5	-0.5	-1.5	-1	-1.5	-1.5
G-24	B	F	0	0	0	0	0	0	0	0	0	0	-0.5	-1	0	0	0	0	0	0
O-17	B	F	0	0	0	0	0	0	0	0	-1	0	-1	-1	-2	-2	-1.5	-1.5	-1.5	-1.5
R-03	B	F	0	0	0	0	0	0	0	0	0	0	-0.5	0	0	0	0	0	-1	-1
G-04	B	M	0	0	0	0	-1	-1	-1	0	0	0	-1	-1	-1	-1	0	0	-1	-1
		Avg.	0.00	0.00	-0.13	-0.13	-0.13	-0.13	-0.13	-0.25	-0.25	0.00	*-0.75	*-0.63	-0.63	‡-0.75	‡-0.75	-0.69	-0.75	*-0.94
		SD	0.00	0.00	0.33	0.33	0.33	0.33	0.33	0.66	0.43	0.00	0.35	0.48	0.65	0.61	0.66	0.61	0.61	0.46
B-15	C	F	0	0	0	0	0	0	0	0	0	0	-0.5	-0.5	0	0	0	-0.5	0	0
O-02	C	F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R-23	C	F	0	0	-0.5	0	-0.5	-0.5	0	0	0	0	0	0	0	0	0	0	0	0
O-07	C	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-1	-1
O-12	C	M	0	0	0	0	-1	0	0	0	0	0	0	0	0	--	0	-2	0	-2
R-08	C	M	0	0	--	–	0	0	0	0	--	–	0	0	--	--	--	–	--	--
R-13	C	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Avg.	0.00	0.00	-0.07	0.00	-0.21	-0.07	0.00	0.00	0.00	0.00	-0.07	-0.07	0.00	0.00	0.00	-0.36	-0.14	-0.43
		SD	0.00	0.00	0.18	0.00	0.36	0.18	0.00	0.00	0.00	0.00	0.18	0.18	0.00	0.00	0.00	0.69	0.35	0.73

(*) Difference from baseline was significantly different than that for controls at $p \leq 0.05$. (‡) Difference from baseline was marginally different than that for controls at $p \leq 0.07$.

Significantly different from baseline time 0 (within group change) with $p \leq 0.05$. Marginally different from baseline time 0 (within group) with $p \leq 0.07$.

Note: The group B average of the left and right sides for weeks 5,6, and 8 were significantly different from the average at time 0 ($p < 0.05$) and at week 7 it was marginally different ($p=0.0625$).

Table 8.4 : Focal sampling data for selected behaviors. Data represent ratios, or proportion of times the behavior occurred out of the total observation time points. A, B, and C are control, 3.5, and 0.5 mg/kg daily dietary MeHg treatment groups. Values are averages ± 1SD (small font numbers), for each treatment at weekly time points (0-8).

Tx	Perching 0	1	2	3	4	5	6	7	8	Standing 0	1	2	3	4	5	6	7	8
A	0.946	0.781	0.563	0.970	0.975	0.858	0.773	0.896	0.782	0.019	0.199	0.279	0.006	0.025	0.073	0.000	0.093	0.182
	0.049	0.227	0.298	0.040	0.049	0.125	0.227	0.179	0.353	0.023	0.216	0.262	0.016	0.049	0.089	0.000	0.183	0.364
B	0.882	0.835	0.835	0.807	0.939	0.889	0.961	0.900	0.684	0.083	0.034	0.145	0.069	0.033	0.111	0.026	0.100	0.309
	0.130	0.207	0.174	0.140	0.085	0.157	0.095	0.200	0.223	0.124	0.060	0.169	0.097	0.053	0.157	0.064	0.200	0.225
C	0.856	0.878	0.753	0.785	0.925	0.891	0.747	0.764	0.747	0.092	0.142	0.149	0.144	0.067	0.006	0.131	0.194	0.245
	0.134	0.141	0.360	0.159	0.130	0.091	0.100	0.175	0.351	0.086	0.185	0.244	0.179	0.133	0.012	0.112	0.159	0.354
	Sleeping 0	1	2	3	4	5	6	7	8	**Looking Around** 0	1	2	3	4	5	6	7	8
A	0.069	0.135	0.000	0.087	0.180	0.256	0.114	0.101	0.000	0.402	0.344	0.340	0.457	0.542	0.197	0.568	0.540	0.506
	0.046	0.110	0.000	0.138	0.213	0.378	0.114	0.107	0.000	0.089	0.205	0.201	0.215	0.283	0.127	0.205	0.198	0.304
B	0.029	0.093	0.227	0.100	0.327	0.323	0.065	0.100	0.068	0.447	0.258	0.327	0.334	0.191	0.303	0.463	0.491	0.498
	0.052	0.120	0.307	0.135	0.392	0.260	0.159	0.200	0.164	0.068	0.212	0.201	0.259	0.204	0.236	0.292	0.429	0.274
C	0.101	0.055	0.159	0.000	0.104	0.188	0.121	0.000	0.156	0.395	0.306	0.455	0.474	0.485	0.273	0.404	0.573	0.522
	0.070	0.097	0.236	0.000	0.166	0.155	0.171	0.000	0.265	0.078	0.276	0.328	0.280	0.265	0.240	0.323	0.334	0.335
	Preening 0	1	2	3	4	5	6	7	8	**Manipulating Objects** 0	1	2	3	4	5	6	7	8
A	0.311	0.231	0.210	0.160	0.152	0.310	0.000	0.273	0.221	0.041	0.155	0.184	0.052	0.022	0.000	0.000	0.010	0.164
	0.119	0.237	0.224	0.161	0.209	0.297	0.000	0.239	0.335	0.063	0.169	0.181	0.095	0.031	0.000	0.000	0.023	0.218
B	0.168	0.284	0.261	0.133	0.070	0.343	0.368	0.209	0.152	0.031	0.068	0.057	0.034	0.027	0.000	0.000	0.000	0.116
	0.077	0.222	0.313	0.155	0.042	0.014	0.357	0.222	0.193	0.042	0.099	0.067	0.063	0.036	0.000	0.000	0.000	0.133
C	0.177	0.389	0.110	0.236	0.277	0.339	0.066	0.188	0.188	0.044	0.090	0.071	0.061	0.018	0.006	0.076	0.085	0.087
	0.036	0.311	0.121	0.275	0.160	0.356	0.056	0.240	0.241	0.048	0.103	0.175	0.068	0.036	0.012	0.057	0.129	0.125

The most common behavior performed while perching was looking around, which the cormorants did about 40% of the time. There were no significant differences between groups in the amounts of time spent looking around. There were also no differences across time as the study progressed. All three groups of birds spent approximately equivalent amounts of time preening, 21-23% of total time. Preening was performed while perching, and all birds were observed preening at some point. There were no significant differences, between groups or across treatment weeks, in the amount of time spent preening. Another behavior which was seen while perching was wing spreading. All three treatment groups engaged in this behavior 5-10% of the time, often after swimming. There were no significant differences across groups or across treatment weeks.

Sleeping was a highly variable activity. In any given week, two to three birds from each group were observed sleeping for some period of time. When comparing weekly averages between treatments there did not appear to be a clear pattern of increasing or decreasing amount of sleep. However, an overall summary, including all times for each group, resulted in averages of 11%, 15%, and 10% sleep time for treatment A, B, and C, respectively. During weeks 4 and 5, group B birds slept about 32% of the time, but in the remaining weeks those high sleep rates returned to normal, or slightly lower, levels. In the mercury-dosed birds, especially group B, fewer birds were observed sleeping during the final weeks of the study, but those that did sleep slept for longer periods of time than previously. Control birds slept for more consistent lengths of time throughout the study than those of the dosed groups.

When birds were not perching, they were either standing or swimming. Groups A and B were observed standing approximately 10% of the time, and group C stood about 13.0% of the time. No increases or decreases were noted as treatment progressed.

During most weeks, three to five cormorants from each group were observed manipulating objects. During week five, no birds from any group were seen performing this behavior, and during week six, only two birds manipulated objects. Objects commonly manipulated included strings from the astroturf flooring, bark from the perches, pine needles, wire from the side of the pen, and fish. Birds tossed the objects into the air, tugged on them, or used them to interact with another bird.

The amount of time spent swimming decreased in all groups during the final weeks of observation. During the first half of the study, three to four birds from each group were usually observed swimming during any given observation period. During the final weeks, zero to one bird from each group swam.

Very little vocalizing was observed, and some weeks none was heard in any group. When vocalizing did occur, usually it was only one or two birds during an observation period. Begging and bathing were the least commonly observed behaviors. Bathing was noted only a total of nine times throughout the entire study. Begging was never seen during an observational period.

The control group perched alone an average of 58% of the time. The low-dose group perched alone 35% of the time, and the high-dose group perched alone 41.5% of the time. Certain individuals perched near one another more often than others. In Pen One, Y-01 and G-04 perched together on about 30% of the observation days, and G-04 and B-05 were together 36% of those days. In Pen Two, Y-06 and R-08 were perching next

to one another on 30% of the observations. Pen Three included O-12 and B-15, who could be found beside one another on 35% of the days. Pen Four did not produce any pairs who consistently perched side-by-side. In Pen Five, which had only three birds, all the birds tended to perched together, with R-23 next to G-24 and B-25 64% and 38% of the time, respectively, and G-24 next to B-25 38% of the observation days.

Interactions between individual birds were measured as the percentage of observation periods on which there was an interaction between any birds. Results were variable, ranging 0 – 29% of days with an interaction between specific penmates. The lowest interactions (0%) were between Y-01 and B-05 and between O-17 and B-20. The highest interaction rates (>20%) were between the following pairs: R-03 and B-05, G-04 and B-05, O-12 and R-13, O-12 and B-15, G-14 and B-15, and G-19 and B-20.

Discussion

There is inherent subjectiveness when assessing the clinical status of an animal. An attempt was made here to reduce personal bias by keeping the study double blinded and standardizing techniques as much as possible. Previous studies involving methylmercury toxicity in birds have shown that the main clinical signs of toxicity include inappetence, loss of body weight, lethargy, fluffed feathers, muscular weakness, ataxia, incoordination, stumbling, and difficulty landing (Borg et al., 1970; Fimreite and Karstad, 1971; Heinz, 1979; Burbacher et al., 1990; Scheuhammer, 1988). None of these signs were overtly apparent in this study of cormorants. Birds remained healthy and alert with no blatant neurological signs. Most reports on the neurotoxicity of MeHg do not describe in detail the specific components of the neurologic assessment. There have been no reports in avian

species describing proprioceptive deficits specifically. It is presumed that with ataxia, incoordination, stumbling, and difficulty landing that proprioception is affected, although it has not been discussed in terms of early detection prior to overt clinical neurological changes. It is likely that the birds in the high-dose group would have soon developed more pronounced neurological impairment had the dosing been carried out further. Perhaps use of proprioceptive testing in future studies would be of value in assessing early effects of methylmercury.

Normal conscious proprioception requires awareness of the position of the extremities and movement without visual input. The sensory component of this process passes through the spinal cord to the brain stem and the somatosensory cortex (Clippinger et al., 1996). A complementary motor component of this pathway is relayed back to the limbs for correction of abnormal placement. A deficit in proprioception may indicate damage to the sensory and/or motor components in peripheral nerves, spinal cord, brain stem, or cerebral cortex.

The effects of MeHg are likely diffuse in nature, and the deficits detected in the cormorant may be due to peripheral as well as central dysfunction. Borg et al. (1970) reported demyelination and nerve cell degeneration in the cerebellar medulla and the medulla oblongata in goshawks fed chickens contaminated with MeHg. Mallard ducks dosed both acutely and chronically with MeHg had lesions in peripheral nerves as well as the spinal cord and the brain. Axons in white matter tracts in the spinal cord were affected, especially the dorsal part of the lateral funiculus (Pass et al., 1975). Several descending motor tracts have been identified in the lateral funiculus (Benzo, 1986).

The finding of proprioceptive deficits in the high-dose group indicated that subtle, early signs of toxicity were developing. The levels of mercury found in the brain and tissues of these cormorants have been associated with overt neurological signs in some avian species (Scheuhammer, 1988). Despite the high levels of mercury found and the early neurological changes, no treatment-related differences were found in the behavioral observations. There were no differences in the proportion of time the birds spent in any one behavior or in the nature or quality of their behaviors. Some minor differences were found, such as the amount of time spent sleeping, but no definitive pattern could be attributed to methylmercury treatment. It was interesting to note that the birds interacted socially in small groups, which did not segregate according to treatment exposure.

Perching was the most frequent behavior among all groups. Perching became less frequent during the second half of the study across all treatment groups. Possibly, as the birds mature, they increased their activity levels and thus perched less. While birds perched, they also looked around, preened, spread their wings, or slept. Vocalization was rare, a finding consistent with observations of wild cormorants. Double-crested cormorants reportedly have a limited array of sounds, with only about nine different sounds of a mostly prolonged or repetitive nature (Johnsgard, 1993). Wing spreading was frequently done after swimming. This was expected, as cormorants are known to spread their wings to allow them to dry.

Begging and bathing behaviors were not seen as frequently as expected. These behaviors were included for observation because they occurred often during preliminary observations. One reason these behaviors may have been reported less frequently than predicted is that they seem to occur in association with feeding (Johnsgard, 1993), but not

between feedings when recording was done. Begging routinely occurred in this study as people arrived to feed.

Bathing usually occurred as soon as the cormorants finished eating. As previously stated, behavioral observations were never done during feeding time, and the observer was not normally involved in the feeding process with these birds. Behaviors typically associated with feeding time were not generally exhibited at other times, and they were not exhibited in response to all people, only individuals who fed them regularly. During three episodes, circumstances prevented the use of the blind, and the observer was clearly visible about 15-20 feet away, but the birds did not respond to the observer's presence in any noticeable way.

Unlike the control group, birds in the low-dose and high-dose groups perched most often near another bird instead of alone. In all three of the groups, when they were perching together, individual birds seemed to have a preference for perching near another specific bird. There did not seem to be any gender association in the preferred perching partnerships. The three birds in Pen Five spent most of their time close to one another, and very little time apart. From these observations, it appeared that a definite social structure was established, and individual preferences and personalities may have accounted for the increased association of individuals. Accounts of behavior in the wild have indicated that cormorant species have highly developed social structures, including group or social feeding strategies (Johnsgard, 1993).

It is interesting to compare the incidence of birds perching side-by-side to the percentage of days with interactions between penmates. It seems logical that the birds which interacted with one another would also be the ones that were perched together, but

this was not the case. Two pairs that were never observed interacting with each other were actually perched together about 20% of the time. Those that had the most interactions were not necessarily the ones that perched together the most. They were perched together ranging from 10% to 36% of the time. In particular, the cormorants in Pen Five, who perched together more than any other pen, had relatively low interaction rates of <17%. It is possible that interactions were more of a territorial nature, but of a relatively non-aggressive character since very few displays of aggression were observed.

In all three groups, interactions decreased during the second half of the study. One reason for this may be developmental, with birds becoming more independent and less social as they aged. In studies with monkeys exposed to methylmercury prenatally, the social behavior in established playgroups showed a significant decrease in play behavior and non-social behavior. Exposed infants spent more time alone (Gilbert and Grant-Webster, 1995). In this study, the effects are unrelated to mercury toxicity, as evidenced by a similar trend within the control group.

As a total group, the cormorants spent slightly more time perched alongside another bird than alone (45.8% of the time alone and 54.2% together). There was a wide range of time that birds perched together or alone, with some being alone 14% of the time and others 76%. Again, it seems that there are individual differences in preferences for proximity to certain birds, but no relationship to mercury dosing could be found.

Different groups of birds did seem to have favorite perching spots. Each pen of birds seemed to prefer certain perches, which were not the same as another pen's preferences. Pen One and Pen Five both contained an outer wall and birds in these pens rarely used the perches on the outer screens, preferring the perches that were closer to an

adjoining pen. Many birds selected the higher perches, specifically G-04, R-23, G-24 and B-25, all of whom seldom returned to a low perch once the higher perches were placed in their pens.

Although many of the activities observed in this study have also been reported in wild cormorants (Johnsgard, 1993), the results presented here cannot be extrapolated, directly to free-ranging cormorants. Captivity certainly affects behaviors, but the current study provides important baseline information and controlled dosing data useful for comparative purposes. Observing larger numbers of birds at more frequent intervals may increase the sensitivity of these tests in detecting toxicity. Possibly the more active and demanding requirements of life in the wild may lead to more apparent behavioral changes due to toxicant exposure. Perhaps, also, behavioral observations are not adequately sensitive for use as an indicator of mild to moderate MeHg exposure and evaluation of other early indicators may be more appropriate. The birds in this study had levels of mercury that have been determined to be toxic in other species, without noticeable behavior changes. It may be that cormorants in the wild are able to behave in ways that are consistent with typical behavioral patterns even though they may still have increased levels of tissue mercury (i.e., a normal behavior pattern does not rule out MeHg exposure).

In conclusion, mild to moderate proprioceptive deficits were detected in the high-dose group starting at week 5, while no significant differences were found in behavioral observations. Behavioral observation, under the given conditions, was not a sensitive technique for detection of early methylmercury toxicity. In contrast, systematic neurological exams may be an effective means of detecting very early functional changes in MeHg toxicity in birds.

CHAPTER 9
HEMATOLOGY

Introduction

Methylmercury can accumulate in high concentrations in blood and it has the ability to bind to a variety of cellular components. Thus, there is the potential for this toxicant to have adverse effects on blood cells as well as other hematologic parameters. Currently, there is limited literature on the effects of MeHg on CBC and serum chemistry values, and reports that do exist vary widely in their results. Methylmercury toxicity in great egret nestlings caused a decrease in PCV, TPP, and lymphocyte numbers and an increase in AST and ALT at a dose of 5ppm for about 12 weeks (Frederick et al., 1997). In mice, methylmercury caused an increase in PCV and no change in white blood cells (Ilback 1991). In rats, MeHg caused no change in CBC values, but caused an increase in BUN and alkaline phosphatase (Verschuuren et al., 1976). The objective of this component of the project was to describe normal hematologic and biochemical parameters for *Phalacrocorax auritus* between the ages of approximately 8 and 16 weeks, and subsequently to identify changes, if any, in these parameters as a result of 8 weeks of MeHg intake at 0.5 or 3.5 mg/kg diet.

Methods

Sampling was conducted at the USDA Wildlife Research Center where the birds were housed, except on weeks 0, 4, and 8, when blood samples were collected at the electrophysiology testing lab while birds were under general anesthesia. Blood was collected by venipuncture of the wing vein or medial metatarsal vein and placed in 3 ml lithium heparin tubes and chilled. Blood smears were made immediately with fresh, non-heparinized blood, air dried, and fixed with methanol. The volume of blood taken (including that saved for Hg analysis) ranged between 2-3 ml, but never exceeded 3 ml per bird per week.

CBCs were evaluated at 2-week intervals and included packed cell volume (PCV), total plasma proteins (TPP), total white cell count (TWBC), total red blood cell count (TRBC), and a differential white blood cell count. Direct counts of total WBCs and total RBCs were performed using a Natt and Herrick's stain (Campbell, 1995) and a Neubauer-ruled hemacytometer. Both TWBC and TRBC were counted on the same hemacytometer after a 1:100 dilution of blood to Natt and Herrick's stain. Blood smears were stained with Fisher Diagnostics Leukostat Stain™ (Fisher Scientific, Pittsburgh, PA), a modified Wrights stain technique, and differential WBC counts were performed including percentages of heterophils, lymphocytes, monocytes, eosinophils, and basophils out of a minimum count of 200 cells.

Serum chemistry panels were evaluated at 4-week intervals (time 0, 4, and 8 weeks). Blood samples were submitted to the University of Florida Veterinary Medical

Teaching Hospital Clinical Pathology Laboratory, and serum was analyzed on a CIBA-CORNING 550 Express Analyzer (Chiron Diagnostic Corporation, East Walpole, MA). Chemistry panels included 20 parameters according to the avian/reptilian protocol used by this laboratory. Data from serum chemistries, CBCs, and differential blood counts (absolute values) were statistically analyzed using repeated measures ANOVA and Tukey-Kramer multiple pairwise comparisons (PROC GLM, SAS 6.1).

Results

CBCs

Results for CBC values and differential cell counts for all treatments are presented in Figures 9.1-9.9. Percentile distributions of the various cell types are summarized in Table 9.1. Packed cell volume was initially low prior to dosing, ranging from about 20 to 30% and increasing to around 45 - 50% when the birds were about 8-9 weeks of age, at which dosing was started (Figure 9.1). Average PCV remained at 45-50% in all groups throughout the remainder of the trial. The TPP was approximately 3.2 to 4.2 g/dl prior to dosing and had a gradual increasing tendency until the end, at which time values were about 4.9-5.0 g/dl for all treatment groups (Figure 9.2). Total RBC count increased, then plateaued at an average of 2.7 - 3.0 x 10^6/µl, at which level it remained for all groups until the end of testing (Figure 9.3). Total WBC, although quite variable, remained within roughly the range of 10 - 18,000 /µl in all treatment groups (Figure 9.4).

There were no clear treatment-related differences in PCV, TPP, total WBC, or total RBC values. Several minor differences were found but were not considered an effect

of MeHg dosing. There was a time by treatment effect in which TPP in group B at time 0 was lower than TPP in B at 5, 6, 7 and 8 weeks ($p<0.001$). The RBC average for group B at time 0 was higher than the control at that time ($p=0.0411$), but this was likely due to an outlier which had and RBC count of 6.05 x 10^6/µl compared to 2.5 -3.95 x 10^6/µl for all other subjects in that group. Consequently, within group B, the RBC average at time 0 was higher than at weeks 1, 4, and 7. These were not, however, thought to be related to dosing because of the unusually high time 0 value in group B.

Differential cell-counts (Figures 9.5-9.9) including heterophils, lymphocytes, monocytes, eosinophils and basophils, had no statistically significant changes over time, except between B2 and B4 lymphocytes ($p = 0.0311$). There were no treatment-related differences between groups at any of the 2-week time points, for any of the cell types. Across all treatment groups there was a trend in WBC percentages, at alternating time points, with heterophils higher on weeks 0, 4, and 8, and lymphocytes and eosinophils lower on those weeks than on weeks 2 and 6 (Table 9.1). Heterophils, on weeks 0, 4, and 8, comprised approximately 50-63% of the total WBC count, while lymphocytes accounted for about 35-45%. On weeks 2 and 6 this trend was reversed. Except for lymphocytes B2 and B4 as noted above, the fluctuating trends were not significant. The same trend held true for absolute cell counts also.

Chemistries

Results for serum chemistry values obtained from control and treatment groups are presented in Table 9.2. Significant ($p \leq 0.05$) changes were seen in the control group (A) over time in several parameters. Increases were seen from week 0 - 4 and 0 - 8 in CO_2,

creatinine, cholesterol, albumin:globulin ratio, and AST. Anion gap and uric acid both decreased significantly from weeks 0 - 4 and 0 - 8. Sodium increased from weeks 4 - 8. Albumin increased from weeks 0 - 8. ALT decreased from weeks 0 - 8. Potassium was markedly increased in week 4, but returned to levels similar to baseline by week 8.

Serum chemistry values, for treatment groups B (3.5 mg/kg) and C (0.5 mg/kg), were similar to the control group, and no significant treatment related differences were apparent. As in the control group, changes over time were noted in a few parameters in both of the MeHg treated groups. Patterns of time-related changes in these groups were consistent with those seen in controls.

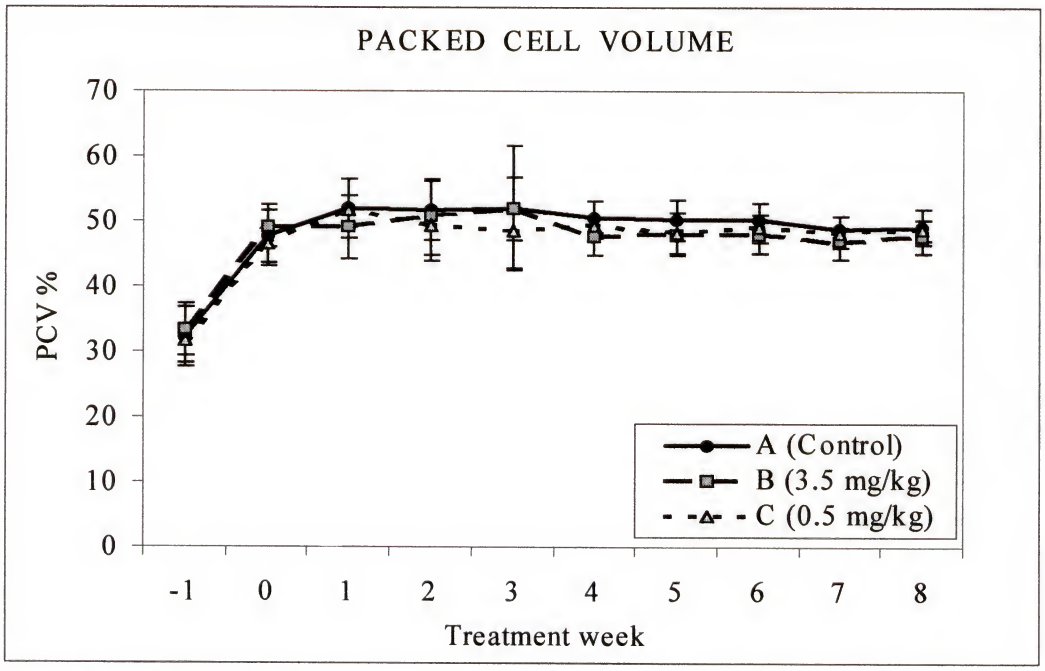


Figure 9.1 : Weekly packer cell volume averages (±1 SD) for controls and two MeHg treatment groups. Week 0 corresponds to approximately 8-9 weeks of age.

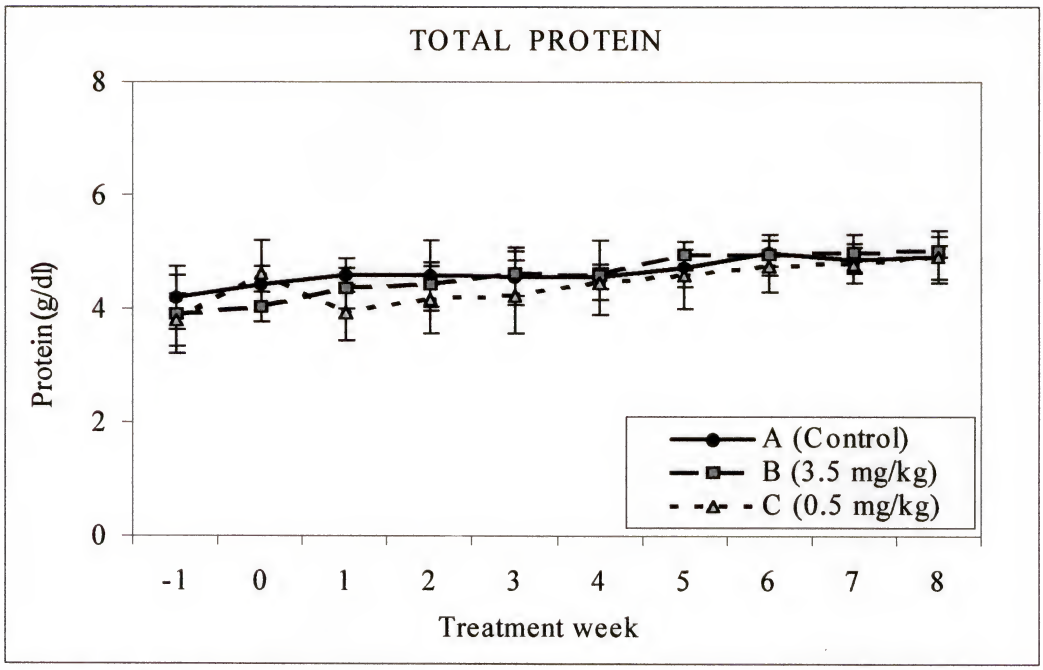


Figure 9.2 : Weekly total protein values for three treatment groups. Points are averages ± 1SD.

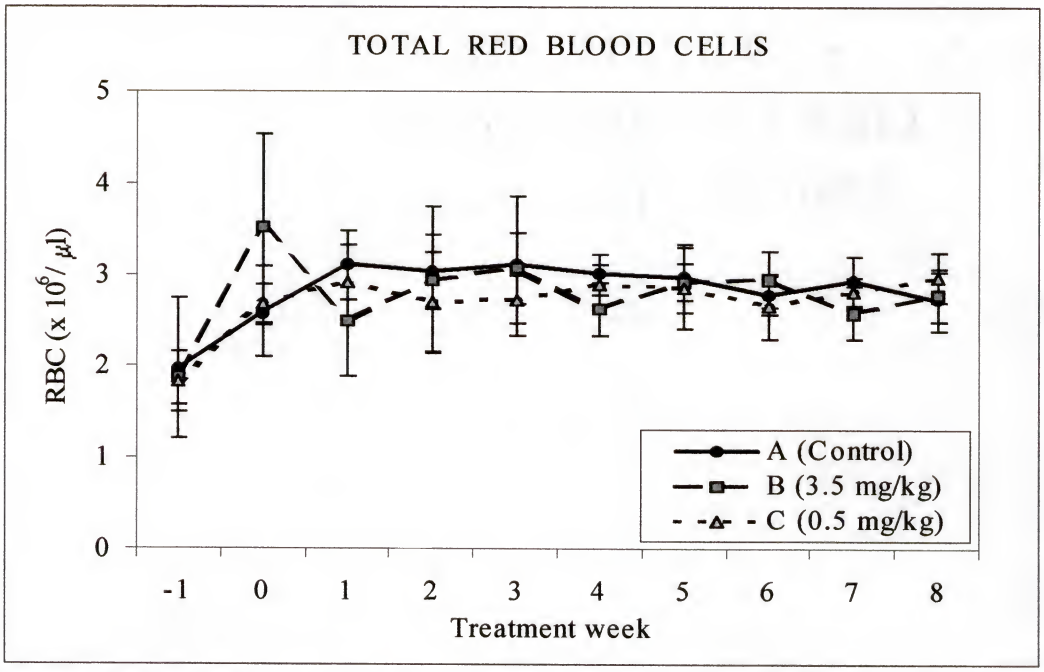


Figure 9.3 : Weekly total red blood cell counts (averages ± 1 SD) for controls and two MeHg treatment groups. Week 0 corresponds to approximately 8-9 weeks of age.

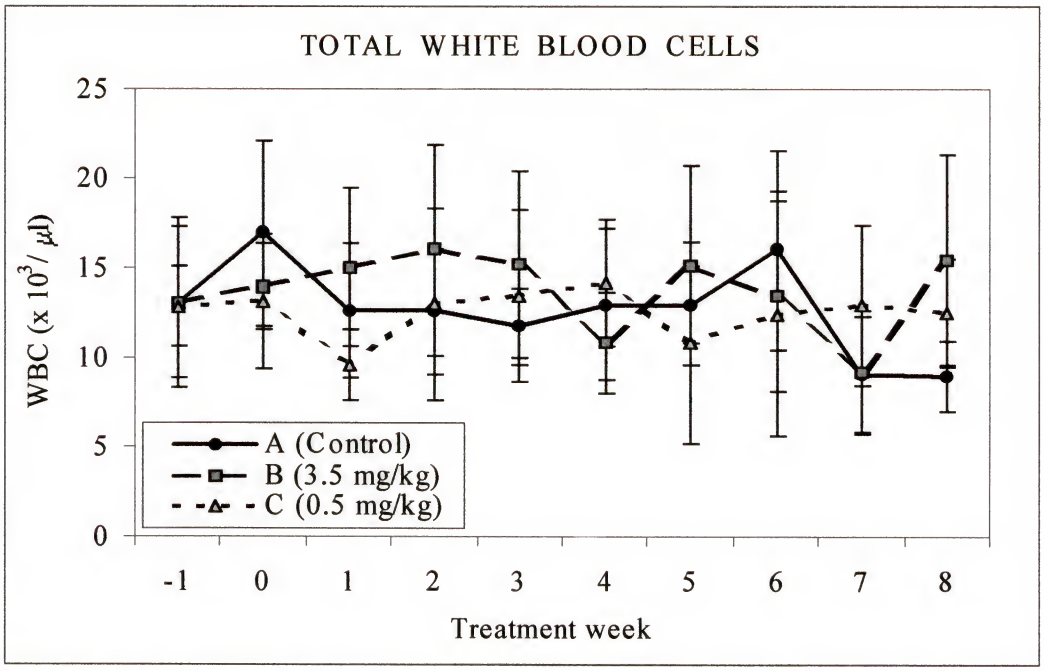


Figure 9.4 : Weekly total white blood cell counts (averages ± 1 SD) for controls and two MeHg treatment groups.

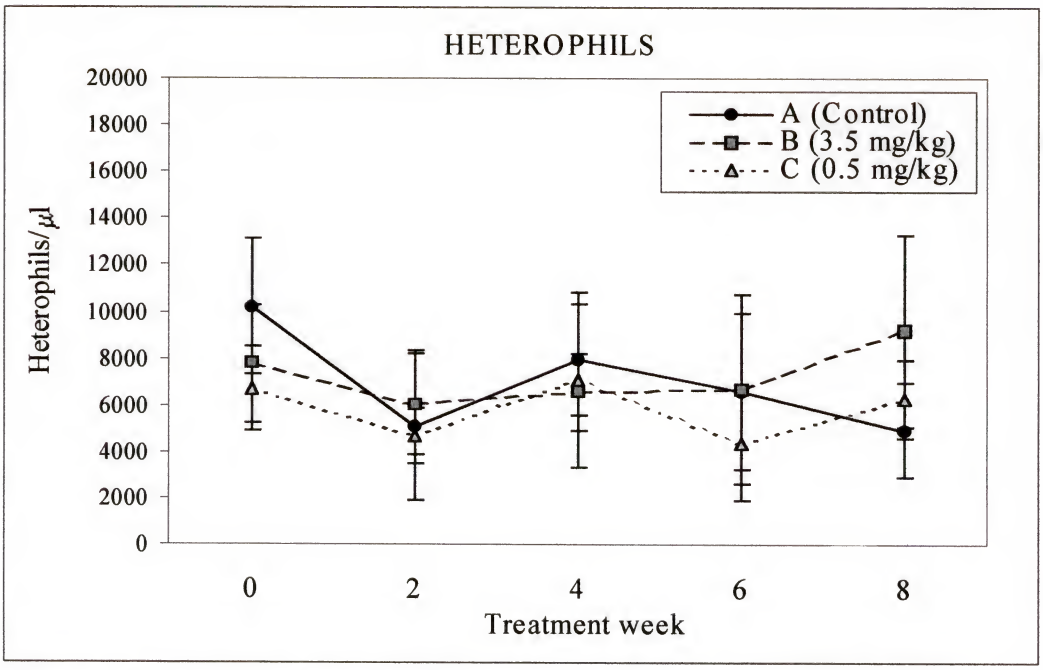


Figure 9.5 : Absolute heterophil count (±1SD) for three treatments of daily methylmercury.

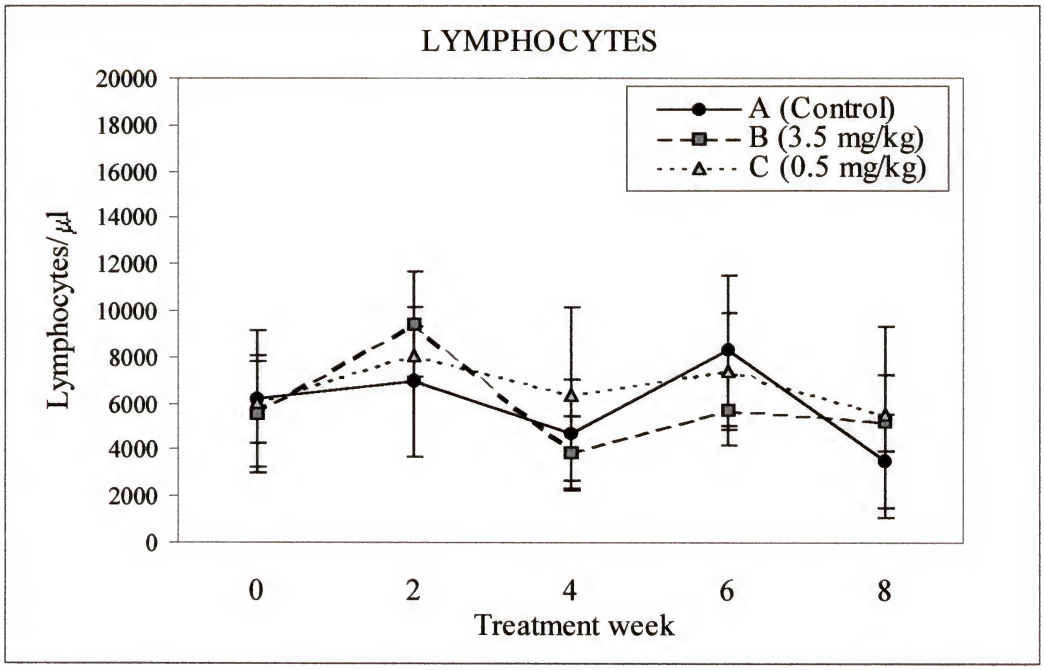


Figure 9.6 : Absolute lymphocyte count (±1SD) for three MeHg treatment groups.

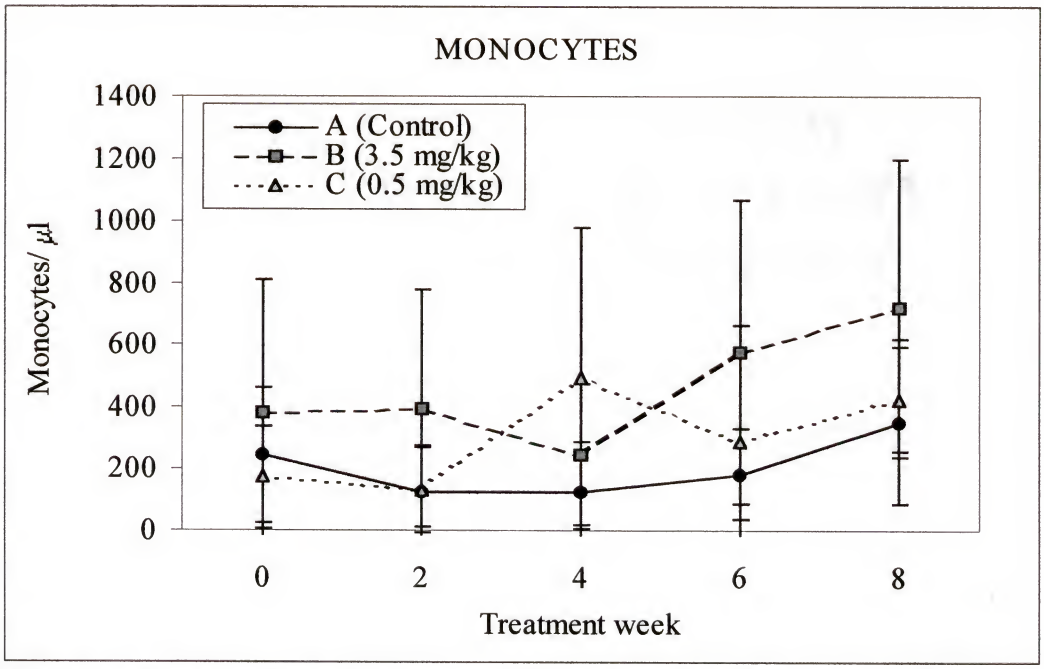


Figure 9.7 : Absolute monocyte count (±1SD) for three MeHg treatment groups.

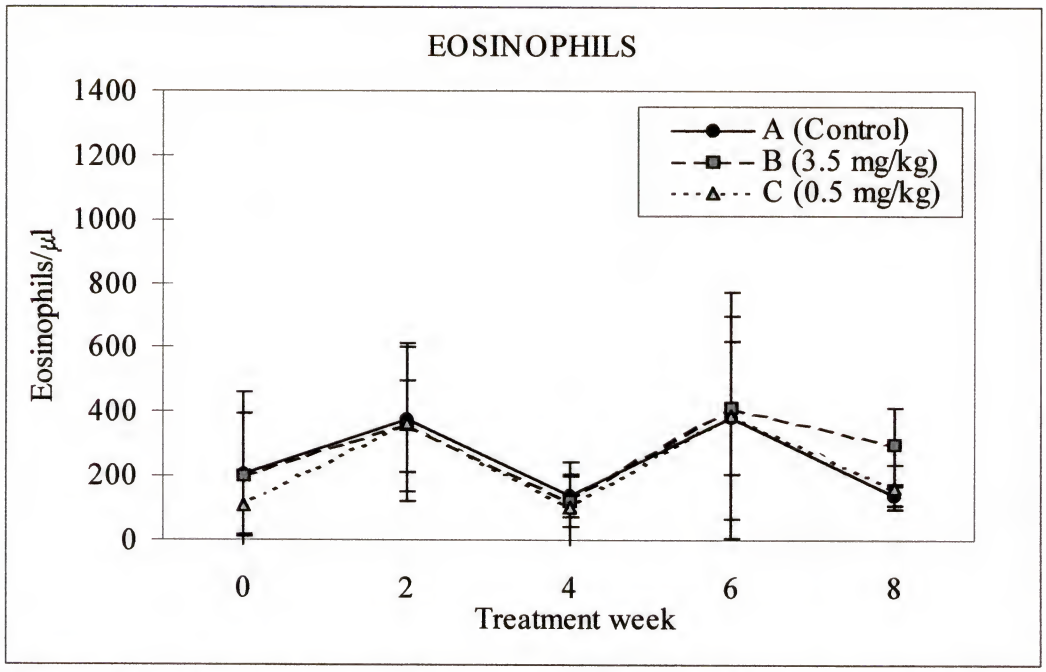


Figure 9.8 : Absolute eosinophil count (±1SD) for three MeHg treatment groups.

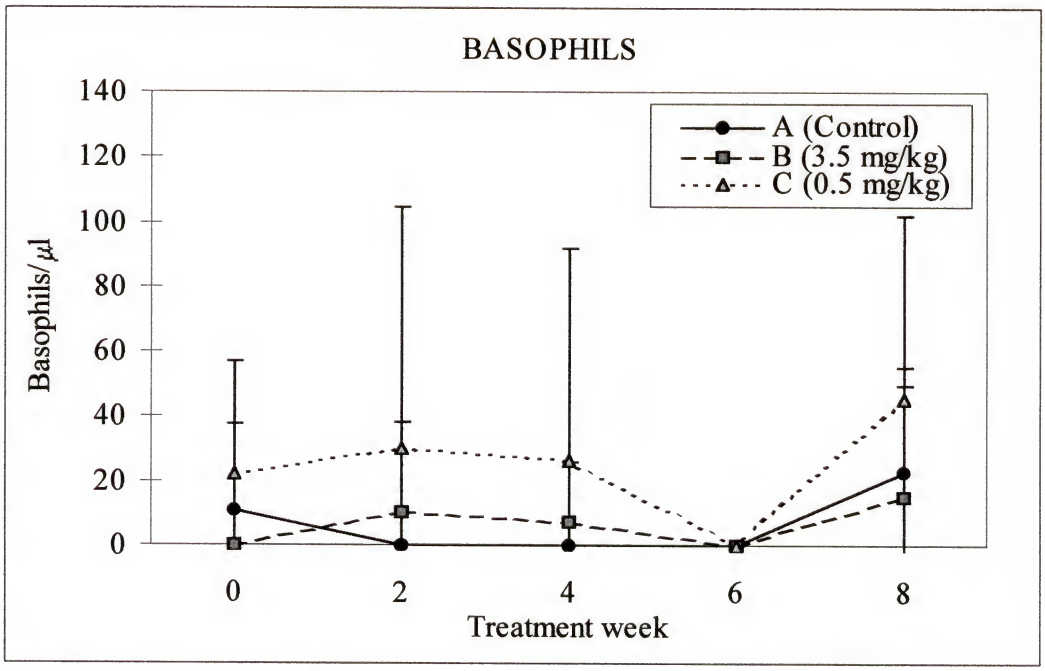


Figure 9.9 : Absolute basophil count (±1SD) for three MeHg treatment groups.

Table 9.1 : Differential white blood cell counts as percentages (± 1SD) of total WBCs. A, B, and C are control, high-, and low-dose methylmercury treatments, respectively.

Cell Type	Tx	Week 0	Week 2	Week 4	Week 6	Week 8
Heterophil (%)	A	61.64 (11.28)	38.00 (12.41)	63.00 (12.09)	43.33 (7.23)	53.93 (13.64)
	B	57.38 (17.34)	39.21 (6.84)	61.56 (7.44)	45.71 (11.67)	58.79 (8.80)
	C	53.14 (13.59)	39.00 (8.88)	51.00 (20.86)	36.64 (6.73)	50.25 (5.22)
Lymphocyte (%)	A	35.71 (12.03)	57.71 (12.65)	34.71 (13.14)	50.50 (10.78)	40.00 (13.76)
	B	38.00 (17.53)	56.07 (6.09)	34.44 (9.71)	45.86 (13.35)	34.45 (9.96)
	C	44.71 (13.75)	60.07 (10.88)	45.03 (21.59)	58.71 (3.73)	44.57 (6.39)
Monocyte (%)	A	1.43 (1.18)	1.14 (1.36)	0.93 (1.08)	1.33 (1.25)	4.14 (2.82)
	B	2.88 (3.25)	2.07 (1.61)	2.81 (3.17)	4.57 (4.52)	4.34 (2.36)
	C	1.21 (1.06)	1.21 (1.25)	3.06 (2.67)	2.07 (2.34)	3.42 (1.40)
Eosinophil (%)	A	1.14 (0.99)	3.14 (1.81)	1.21 (0.65)	2.33 (0.94)	1.64 (0.52)
	B	1.38 (1.60)	2.57 (1.40)	1.13 (0.65)	3.86 (1.94)	2.05 (0.88)
	C	0.79 (0.65)	2.64 (1.09)	0.77 (1.00)	2.57 (1.92)	1.41 (0.73)
Basophil (%)	A	0.07 (0.17)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.29 (0.36)
	B	0.00 (0.00)	0.07 (0.17)	0.06 (0.17)	0.00 (0.00)	0.13 (0.33)
	C	0.14 (0.23)	0.29 (0.70)	0.14 (0.35)	0.00 (0.00)	0.36 (0.44)

Table 9.2 : Serum chemistries for three treatment groups at three times. A = control, B= 3.5 ppm, C = 0.5ppm.

		Week 0					Week 4					Week 8				
	Tx	**Avg.**	n	SD	Min	Max	**Avg.**	n	SD	Min	Max	**Avg.**	n	SD	Min	Max
Sodium	A	**142.1**	7	2.5	137.7	146.1	**138.1**	7	6.7	126.1	149.1	**146.0**	7	2.5	141.9	150.3
(mEq/L)	B	**142.4**	8	4.4	139.5	154.0	**144.1**	8	5.6	134.0	149.9	**149.9**	8	3.9	143.5	155.8
	C	**141.0**	7	1.2	138.9	142.7	**143.4**	7	6.6	132.8	152.8	**150.5**	7	4.6	145.0	157.3
Potassium	A	**5.3**	7	1.8	3.1	7.8	**12.2**	5	4.4	4.1	16.7	**6.4**	7	1.7	4.0	8.7
(mEq/L)	B	**5.6**	8	1.2	4.5	8.3	**7.3**	7	4.8	3.6	17.0	**6.0**	8	2.2	3.3	10.0
	C	**4.8**	7	0.5	4.2	6.0	**9.5**	6	6.0	3.1	18.1	**5.0**	7	1.6	3.6	7.7
Chloride	A	**113.1**	7	4.3	104.0	117.0	**115.9**	7	1.1	114.0	117.0	**114.3**	7	1.8	111.0	117.0
(mEq/L)	B	**113.6**	8	3.0	111.0	121.0	**114.6**	8	1.4	112.0	116.0	**115.3**	8	1.3	113.0	117.0
	C	**112.6**	7	2.9	106.0	116.0	**115.1**	7	2.2	112.0	118.0	**113.9**	7	2.0	111.0	117.0
Carbon	A	**21.7**	7	2.8	15.9	24.7	**36.2**	7	3.1	30.5	39.1	**34.7**	7	3.1	31.1	38.9
Dioxide	B	**22.4**	8	3.7	17.0	28.4	**35.7**	8	2.7	31.6	40.1	**36.5**	8	2.8	32.4	40.7
(mEq/L)	C	**21.7**	7	1.8	18.0	24.2	**35.7**	7	4.6	28.4	41.9	**35.9**	7	3.2	30.3	39.9
Anion Gap	A	**12.5**	7	3.3	7.3	16.4	**1.7**	3	1.2	0.1	2.7	**4.0**	6	1.5	1.2	6.2
(mEq/L)	B	**12.0**	8	5.1	5.7	22.8	**3.3**	6	2.1	0.5	6.1	**6.2**	6	3.9	1.9	12.2
	C	**11.6**	7	2.8	7.7	17.1	**3.0**	6	1.9	0.1	6.3	**5.7**	7	2.7	3.0	10.6
BUN	A	**4.4**	7	4.9	1.3	16.1	**3.7**	6	0.9	2.2	5.0	**2.7**	7	0.8	2.0	4.7
(mg/dL)	B	**2.2**	8	0.4	1.6	3.0	**2.5**	8	0.4	1.9	3.0	**2.7**	8	0.7	1.9	3.9
	C	**2.7**	7	0.7	1.3	3.6	**3.0**	7	0.6	2.1	3.8	**2.8**	7	0.6	1.9	4.0
Creatinine	A	**0.20**	7	0.05	0.10	0.30	**0.35**	6	0.05	0.30	0.40	**0.33**	7	0.05	0.30	0.40
(mg/dL)	B	**0.25**	8	0.05	0.20	0.30	**0.36**	8	0.05	0.30	0.40	**0.37**	8	0.04	0.30	0.40
	C	**0.21**	7	0.03	0.20	0.30	**0.35**	6	0.08	0.30	0.50	**0.36**	7	0.05	0.30	0.40
Calcium	A	**10.8**	7	0.8	9.3	12.2	**9.9**	6	0.3	9.4	10.3	**10.1**	7	0.5	9.4	11.2
(mg/dL)	B	**11.1**	8	1.2	9.9	14.0	**9.5**	8	0.3	9.0	10.0	**9.5**	7	0.3	8.9	9.9
	C	**10.5**	7	0.4	10.0	11.1	**9.9**	8	0.6	8.6	10.6	**10.0**	7	0.5	9.3	10.7
Glucose	A	**182**	7	28	141	231	**204**	6	15	183	228	**226**	7	21	201	271
(mg/dL)	B	**212**	8	37	163	274	**203**	8	7	190	214	**202**	8	24	168	246
	C	**177**	7	27	118	205	**228**	7	20	209	275	**217**	7	26	193	269
Phosphorus	A	**7.2**	7	1.7	5.1	10.7	**7.2**	6	0.6	6.1	7.9	**6.8**	7	0.4	6.5	7.6
(mg/dL)	B	**7.4**	8	2.1	2.8	10.8	**6.9**	8	1.1	5.4	9.2	**6.4**	8	0.8	5.5	7.9
	C	**7.0**	7	2.1	4.5	11.9	**6.7**	6	1.0	5.4	8.4	**7.0**	7	0.9	5.9	9.1

Table 9.2 : (continued)

	Tx	Week 0					Week 4					Week 8				
		Avg.	n	SD	Min	Max	**Avg.**	n	SD	Min	Max	**Avg.**	n	SD	Min	Max
Total	A	**0.51**	7	0.31	0.20	1.10	**0.77**	6	0.23	0.50	1.20	**0.73**	7	0.29	0.40	1.10
Bilirubin	B	**0.68**	8	0.16	0.40	0.90	**0.66**	8	0.16	0.50	1.00	**1.05**	8	0.21	0.80	1.40
(mg/dL)	C	**0.66**	7	0.29	0.20	1.00	**0.75**	6	0.44	0.00	1.50	**0.60**	7	0.23	0.30	1.00
Cholesterol	A	**200.4**	7	38.3	154.1	275.6	**274.8**	6	39.2	230.8	344.1	**295.1**	7	46.8	249.2	366.6
(mg/dL)	B	**184.9**	8	20.7	161.9	231.9	**273.4**	8	32.3	239.7	330.5	**322.9**	8	23.7	299.9	375.4
	C	**198.6**	7	28.7	155.5	242.2	**268.0**	6	59.1	216.3	389.5	**312.6**	7	56.8	233.9	388.6
Uric Acid	A	**12.3**	7	5.04	7.1	22.3	**5.8**	6	0.56	5.0	6.4	**4.4**	7	1.29	3.0	7.0
(mg/dL)	B	**11.1**	8	2.50	7.5	16.1	**5.6**	8	1.22	4.7	8.4	**4.3**	8	1.27	2.4	6.4
	C	**11.6**	7	3.06	5.8	15.0	**5.9**	7	1.56	3.7	8.2	**4.9**	7	1.80	2.5	8.4
Total	A	**4.2**	7	0.44	3.6	5.1	**4.0**	6	0.38	3.4	4.5	**4.3**	7	0.34	3.8	4.9
Protein	B	**4.3**	8	0.71	3.5	5.4	**4.0**	8	0.27	3.4	4.3	**4.5**	8	0.11	4.3	4.7
(g/dL)	C	**4.0**	7	0.29	3.7	4.5	**4.2**	6	0.45	3.6	4.9	**4.3**	7	0.41	3.6	4.8
Albumin	A	**1.1**	7	0.16	0.9	1.3	**1.2**	6	0.09	1.1	1.4	**1.4**	7	0.15	1.3	1.7
(g/dL)	B	**1.2**	8	0.15	0.9	1.4	**1.3**	8	0.11	1.1	1.4	**1.6**	8	0.12	1.4	1.8
	C	**1.1**	7	0.07	1.0	1.2	**1.4**	6	0.27	1.0	1.7	**1.6**	7	0.16	1.3	1.7
Globulin	A	**3.0**	7	0.34	2.6	3.8	**2.8**	6	0.34	2.3	3.3	**2.8**	7	0.23	2.5	3.2
(g/dL)	B	**3.2**	8	0.64	2.3	4.2	**2.6**	8	0.16	2.3	2.8	**2.8**	8	0.11	2.6	3.0
	C	**2.9**	7	0.29	2.5	3.3	**2.8**	6	0.26	2.6	3.3	**2.8**	7	0.27	2.3	3.1
Albumin/	A	**0.37**	7	0.05	0.30	0.40	**0.48**	6	0.04	0.40	0.50	**0.50**	7	0.00	0.50	0.50
Globulin	B	**0.38**	8	0.08	0.30	0.50	**0.51**	8	0.03	0.50	0.60	**0.58**	8	0.04	0.50	0.60
Ratio	C	**0.41**	7	0.03	0.40	0.50	**0.48**	6	0.07	0.40	0.60	**0.59**	7	0.03	0.50	0.60
ALP	A	**2122**	7	614	1165	2925	**2376**	6	631	1332	3200	**2266**	7	499	1254	2820
(U/L)	B	**2425**	8	553	1690	3430	**2249**	8	827	1195	3590	**1678**	8	699	1198	3360
	C	**3001**	7	866	2235	4780	**2600**	7	1007	1342	4460	**1965**	7	864	1250	3835
SGPT/ALT	A	**191**	7	33	142	254	**155**	6	20	129	184	**126**	7	31	80	173
(U/L)	B	**175**	8	29	122	228	**146**	8	27	125	211	**120**	8	33	81	195
	C	**197**	7	29	138	235	**116**	7	34	39	155	**114**	7	12	96	133
SGOT/AST	A	**212**	7	12	198	239	**465**	7	49	398	529	**463**	7	62	399	555
(U/L)	B	**204**	8	24	173	241	**479**	8	68	373	617	**502**	8	121	377	793
	C	**223**	7	55	154	305	**460**	7	71	390	582	**440**	7	58	383	533

Discussion

The values for CBC and differential counts which we obtained from the control group birds are comparable to values for other juvenile birds and similar species (Fairbrother and O'Loughlin, 1990; Balasch et al.,1974; Clubb et al., 1991; Woerpel and Rosskopf, 1984). Although no significant changes were seen over time in the CBC or differential counts, we did identify a trend in some of the WBC parameters which consisted of a relative heterophilia, lymphopenia, and eosinopenia on weeks 0, 4, and 8 compared to weeks 2 and 6. A possible explanation for this trend could be that this is a stress response due to handling. On weeks 0, 4, and 8, the blood was collected after inducing anesthesia for ocular testing, which required transport to the VMTH and additional handling compared to weeks 2 and 6. However, a similar oscillation of CBC differential values was seen in a mercury study conducted in great egrets (Frederick et al., 1997). In that study, the changes in cell counts were proposed to be caused by blood collection. It is also possible that these oscillations represent normal variances seen in these birds. Due to the fluctuations of heterophils and lymphocytes, no clear statement could be made regarding the predominant cell type. In some avian species lymphocytes are more numerous, while in others, heterophils predominate (Ritchie et al., 1994).

The chemistry values obtained from the control group birds are also very similar to values for other avian species, as well as for other juvenile birds (Frederick et al., 1997; Clubb et al., 1991; Woerpel and Rosskopf, 1984; Gascoyne et al., 1994). Although averages for many of the serum chemistry parameters had significant changes over time, the values at all time points were still within normal ranges for other avian species. Thus,

these changes were viewed as normal developmental variations and no particular clinical importance was attached to them. The peak noted in potassium at week 4 was present in all 3 treatment groups and rose to a value which is incompatible with life; therefore we account this change to either a collection, storage, or handling error (Woerpel and Rosskopf, 1984; Fudge, 1997; Hochleithner, 1994).

Uric acid was very high on week 0, but by week 4 was within normal limits for other species. Normal uric acid levels are generally between 2-14 mg/dl and increases have been reported as a normal finding in birds of prey, and in cases of lipidemia, dehydration, tissue damage, and starvation (Fudge, 1997; Hochleithner, 1994; Harris, 1991).

Carbon dioxide, in the present study, increased to 30-40 mEq/l in weeks 4 and 8 in all groups (laboratory reference range for avian species = 21-26 mEq/l). A clear explanation for this apparent metabolic alkalosis was not available. However, diving bird physiology may vary considerably from that of other birds. Research has indicated that some diving birds normally have a higher blood CO_2 and blood bicarbonate concentration than other avian species due to their regular conversion to anaerobic metabolism during dives (Lefant et al., 1998; Butler and Jones, 1997). Ludders et al. (1995) reported that masking a diving bird, as when inducing anesthesia, can stimulate their diving reflex, setting these metabolic changes in motion. The high values detected for ALP are seen in juveniles and are commonly due to its wide tissue distribution and the high rate of growth, especially bone growth, in juveniles (Woerpel and Rosskopf, 1984; Fudge, 1997; Hochleithner, 1994). ALT, although higher than normal for most species, is nonspecific in avian species (Fudge, 1997; Hochleithner, 1994; Harris, 1991).

Although there are species differences in normal blood cells, the cormorant's blood cells are fairly characteristic of most avian species (Campbell, 1995). The heterophils have grey to pink rod-shaped granules, whereas the eosinophils have very distinct round pink granules. Lymphocytes in this species appear extremely variable in size and subjectively seem to get larger as the birds get older. Large lymphocytes were sometimes extremely difficult to differentiate from monocytes. Studies of monocyte and lymphocyte counts done by electrophoresis and compared to manual counts have shown that manual counts slightly underestimate monocyte numbers because of difficulties in differentiation. Additionally, in many cases, no monocytes are reported (Woerpel and Rosskopf, 1984; Fudge, 1997). Thus, our monocyte counts, performed manually, may be slightly inaccurate, and further analysis would be necessary to obtain more accurate counts. Basophils are also frequently absent from peripheral blood smears (Fudge, 1997), as was the case in this study.

High levels of mercury have been identified in the tissues and blood of double-crested cormorants in south Florida (Sepúlveda et al., 1998). This increase has been thought to be associated with a high disease prevalence seen in birds from this area. Because clinical pathology can be a sensitive indicator of disease, it was thought that changes induced by mercury, which could lead to an increase in disease susceptibility, might be identifiable in this manner. The results presented here, however, indicate that hematological and biochemical parameters are not affected at the exposure levels tested, suggesting that these parameters may have limited use as subclinical monitoring tools for methylmercury.

No statistically significant effects due to mercury dosing were seen in any of the blood parameters measured in this experiment, although previous experiments have noted some changes (Frederick et al., 1997; Ilback, 1991; Verschuuren et al., 1976). Methyl-mercury toxicity in great egret nestlings caused a decrease in PCV, TPP, and lymphocyte numbers and an increase in AST and ALT (Frederick et al., 1997). In mice MeHg caused an increase in PCV and no change in white blood cell numbers (Ilback, 1991). In rats methylmercury caused no change in CBC values but caused an increase in BUN and alkaline phosphatase (Verschuuren et al., 1976). It is possible that mercury does not have detectable effects on the clinical pathology of cormorants, or that the parameters that it does affect were not measured in this study. In one study conducted on chicks, susceptibility to pathogens varied depending on the time of challenge relative to mercury dosing (Hill, 1980). Therefore, detection of immune-related effects may be dependent on an appropriate challenge at a particular time point. Another possible explanation is that the experiment was not carried out for a long enough time period, or at high enough dose levels, to elicit a response. The effects of mercury on disease susceptibility may be related to the length of time mercury is present in the tissues as well as to its concentration in those tissues. The effects of mercury could also be age dependent in this species.

An aspect of MeHg poisoning not addressed in this study was its interaction with selenium. Selenium can counter the toxic effects of methylmercury (Wilber, 1980). It is possible that the birds in our study had levels of selenium in their bodies that were protective and counteracted any results we may have otherwise seen. Analysis of selenium levels would be necessary in order to further speculate on these types of interactions.

In conclusion, despite reaching levels of approximately 40 ppm mercury in the blood of high-dose birds, no treatment effects were noted on any part of the CBC, the differential WBC count, or the serum chemistries. Values obtained from the control birds in this study are similar to those of other similar avian species. Care must be taken in handling of birds to avoid potential stress-related alterations in differential cell counts which may confound the interpretation of results.

CHAPTER 10
SUMMARY AND CONCLUSIONS

Twenty-two juvenile double-crested cormorants, assigned to one of three groups, were dosed with 0.0 (group A, control), 0.5 (group C), or 3.5 (group B) mg MeHgCl/kg fish per day for 8 weeks. The cormorants remained healthy during the entire study and showed no overt neurological deficits during daily observations. Multiple endpoints were evaluated for changes, and tissue Hg levels were measured. Treatment outcomes are briefly summarized below. Treatment groups are indicated in parentheses.

- Total MeHg intake (mg per animal), including background level in fish, was 0.46 ± 0.04 (A), 8.42± 0.98 (C), and 50.00 ± 3.29 (B) for the 8-week period. Background mercury in fish was 0.2367 mg/kg for sardines.
- There were no effects on food consumption or body weights.
- There were no effects on any part of the CBC, differential WBC, or serum biochemistry panel.
- There were no changes noted in systematic behavioral observations which could be clearly linked to MeHg.
- Weekly neurological exams revealed a pattern of proprioceptive deficits in the high-dose group, starting at week 5. No other neurological tests were altered consistently.

- ERG and VEP waveforms were reproducible over time. The ERG b-wave amplitudes decreased slightly in all treatment groups by week 8 of treatment. The Naka-Rushton model fit the ERG b-wave amplitude profiles very well.
- No treatment-related changes were seen in any ERG parameters or in any of the Naka-Rushton parameters.
- The VEP P1 amplitude was significantly decreased compared to controls on weeks 4 and 8 in the high-dose birds, mainly at the OT1-Ch electrode placement at a medium light intensity. A similar decreasing trend in amplitudes was noted, at high light intensity, for P1 at OT1-Ch, OT2-Ch , and SC-Ch electrode placements, and N1 at OT1-Ch. Changes in the latter points, however, were not statistically significant, or only marginally so.
- Funduscopic exams were normal. IOP measurements tended to be slightly higher in the eyes treated with mydriatics, but no relation to MeHg was noted.
- Gross pathology was unremarkable except for several incidental lesions which were unrelated to MeHg treatment.
- Mercury accumulated rapidly and linearly in tissues, achieving high levels in the 3.5 mg/kg group.
    - Brain average Hg content was 2.4 μg/g and 11.1 μg/g for group C and group B, respectively.
    - Liver average Hg content was 9.8 μg/g and 58.5 μg/g for group C and group B, respectively.
    - Kidney, spleen, and muscle Hg concentrations were between 1-4 μg/g and 20 - 30 μg/g for group C and group B, respectively.

- Fecal-urate Hg concentrations were relatively low, and at higher doses quite variable. In the low-dose group fecal-urate levels were about 0.06 - 0.08 µg/g from week 2 till the end, while the high-dose group reached 1.1 ± 0.8 µg/g by week 8. Control fecal-urate levels remained very low, with averages of 0.011 - 0.017 µg/g for all weeks.

- No histological lesions were seen in liver, kidney, or spleen, and no differences in organ weight were detected.
- Ocular histopathology at the LM level in the high-dose group included vacuolation of the NFL and derangement of the OPL, especially peripherally. On EM, swollen photoreceptor outer segments and fragmented disc lamellae were also apparent, and changes were noted in the IPL in groups B and C, and in the GCs of group C.
- The main neurological findings in the high dose-group included sciatic nerve lesions characterized by swollen myelin sheaths with digestion chambers containing eosinophilic debris (Wallerian degeneration) and Schwann cell hyperplasia. There was axonal degeneration in the cervical spinal cord and swollen ependymal cells in the brain. Other, less-pronounced findings included occasional neuronal degeneration in the spinal cord and a diffuse gliosis throughout the brain, especially in the cerebrum.
- Mercury histo-localization
  - In ocular tissues, mercury was localized in all layers but was most pronounced in the OPL, IPL, NFL, and in the nonpigmented ciliary epithelium.

- In the liver mercury localized along the canalicular margin in the hepatocyes of both the low- and high-dose groups.
- In the brains of high-dose birds there was diffuse staining for mercury in all areas, but large neurons tended to accumulate more, and several areas tended to stain more heavily. The neuropil had very light diffuse staining for mercury throughout all regions of the brain, and glial cells stained but not particularly intensely.
- The region that stained most intensely was neurons of the nucleus isthmi pars magnocellularis. Some of the other areas that had relatively intense staining included the stratum griseum centralle, ependymal cells lining the ventricles, nucleus rotundus, nucleus ruber, nucleus mesencephalicus lateralis, pars dorsalis, nucleus isthmi pars parvocellularis, and Purkinje cells of the cerebellum.

In conclusion, VEPs were useful in detecting early preclinical MeHg toxicity in double-crested cormorants, but ERGs and Naka-Rushton parameters were not. It is possible that ERG changes may have been detected if the study had been continued for a longer period of time or at a higher dose, but in that case it is also likely that overt neurological signs would also have become apparent. Results in the cormorants indicated that early visual system deficits did occur, and that they were most likely centrally mediated. Deficits in the VEPS indicate a lesion, or toxic effect, in the post-retinal visual pathways from the ganglion cells to the cortex, although precise localization of the most affected site cannot be determined by this technique.

While no significant differences were detected between treatment groups using the flash ERG and Naka-Rushton parameters, retinal histopathology was detected. This apparent discrepancy may reflect a lack of sensitivity of these tests at very early stages of toxicity, particularly if receptors in the retina are affected unevenly. Retinal lesions tended to be located more peripherally, but some photoreceptors were also affected in central locations which were viewed by EM. The presence of changes in retinal morphology suggests that there may also be a peripheral component to the visual effects of MeHg in this species.

The visual and other sensory regions in the brains of cormorants tended to accumulate significant amounts of mercury, as evidenced by the heavy staining of selected areas with the autometallography silver-enhancement method. In spite of a functional deficit (decreased VEP amplitude) in the CNS, as well as high levels of mercury, there was little pathology in the brain. The proprioceptive deficits, on the other hand, reflected the neurological lesions which were apparent in the peripheral nerves. It is likely that MeHg impacted both peripheral and central portions of the nervous system concurrently.

The high-dose group accumulated high levels of Hg in blood and tissues, levels which in some avian species have been associated with overt neurotoxicity and death. By comparison, the cormorants of this study appeared healthy, without overt clinically abnormal signs. VEP and proprioceptive deficits indicated early, subclinical toxicity at 4-5 weeks of dosing at the high level. Fecal-urate Hg content was variable, especially at higher doses, but when blood levels were below 25 μg/ml there was fairly good linear correlation. While accurate assessments of MeHg intake based on fecal-urate Hg levels

may not be possible at this time, this data may have use as a monitoring tool to categorize relative exposure levels in wild birds.

Given these findings, a level of 3.5 mg MeHg/kg diet/day or greater could pose a significant risk of overt neurotoxicity to cormorants in the wild, especially for a duration of greater than 4-5 weeks. The low-dose level of 0.5 mg/MeHg/kg would not likely pose a threat of overt neurotoxicity in cormorants, but it has led to tissue Hg levels which have been associated with reproductive and behavioral deficits in other birds.

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BIOGRAPHICAL SKETCH

The path to this point has been varied, interesting, and affected as much by serendipity as it has been by the myriads of people who have touched my life. I was born in Seattle, Washington, and have lived in a number of places including Minnesota, Israel, and Florida. My interests in wildlife, the environment, and toxicology have been with me as long as I can remember and have influenced my career choices along the way.

In 1988 I completed a B.S. in Zoology at the University of Florida (UF).While attending the undergraduate program, I also worked in a variety of different positions including the Marine Biology Department, the Florida Museum of Natural History, and the Office of the Registrar. After graduation I worked at the UF Poultry Science Department for over a year.

While attending the College of Veterinary Medicine, I utilized every opportunity to further my experience in wildlife and avian medicine, aquatic animal medicine, and environmental toxicology. During that time I completed an externship at Care and Rehabilitation of Wildlife (C.R.O.W.) on Sanibel Island, Florida, attended an intensive short-course in aquatic animal medicine and environmental toxicology (ENVIROVET) in Duluth, Minnesota, and attended extra elective wildlife clinical rotations and the special aquatic animal medicine course at UF. Additionally, I was involved in research on manatee neuroanatomy and computerized three-dimensional rendering of unique cell clusters in the

manatee cerebral cortex. I received my D.V.M. degree from the University of Florida in 1993 and subsequently worked in a private small animal practice in Lutz, Florida, prior to returning to UF to pursue a Ph.D. degree in 1994. The program I selected allowed me to combine my interests in environmental toxicology, wildlife and avian medicine, neurology and ophthalmology. It has also provided training in pathology and other diagnostic techniques which I have also enjoyed.

I enjoy the outdoors immensely, and when I am not working I spend much of my time observing wildlife and nature, and sharing what I find with my husband, Gary. Each day is an opportunity to see and learn something new, and to hear another good joke and laugh.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Don A. Samuelson, Chair
Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Roger Reep, Cochair
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Veterinary Medicine and to the Graduate School and was accepted as partial fulfilment of the requirements for the degree of Doctor of Philosophy.

May 1999

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